JOURNAL OF THE
NATIONAL CANCER INSTITUTE

VOLUME 4 *August 1943* NUMBER 1

An Adaptation of the Transparent-Chamber Technique to the Mouse

By GLENN H. ALGIRE,[1] *National Cancer Institute, National Institute of Health, United States Public Health Service*

The transparent-chamber technique for the study of living tissue was developed first by Sandison (*1*, *2*), later perfected and extended by Clark and his collaborators (*3*), and modified recently by Ebert, Florey, and Pullinger (*4*). These workers restricted themselves almost exclusively to animals having sufficiently large ears, such as the rabbit or the dog. Williams (*5*), however, described an adaptation of the transparent chamber to a skin flap in the rabbit and suggested the use of flaps for application of the method to other animals. This suggestion has been followed in the present paper in the adaptation of the method to the mouse.

Of the several types of chambers developed for the work, the one selected was the "preformed tissue" type of Clark et al. (*3*), in which either a single- or double-walled window is in contact with a preformed layer of vascular tissue. Since the previous workers gave complete descriptions of the construction of their chamber and of its insertion into the ear of the rabbit, only such alterations will be described here as were necessary in adapting it to the mouse.

The major problems in this connection were as follows: (1) To produce a skin flap in the mouse sufficiently large to retain a chamber which would be accessible to observation with any objective of the compound microscope; (2) to determine the maximum size of chamber which could be inserted into a skin flap in the mouse and to decide upon the necessary dimensions of each of its parts; (3) to devise methods of immobilizing the unanesthetized mouse and of supporting the chamber on the mechanical stage of the microscope; (4) to adapt methods for transillumination of the chamber which would permit the use of high-power objectives of the compound microscope; and (5) to provide a means of rapid interchange of the stereoscopic and compound microscopes.

THE TRANSPARENT CHAMBER

Two varieties of the preformed tissue chamber were designed, as indicated in figure 1, *A* and *B*. They differ only in that the latter is equipped with a removable top which provides access to the contained living tissue.

Figure 1, *A*, shows the type used when the tumor implant is made at the time of operation. The dimensions are indicated

[1] *Fellow, Finney-Howell Research Foundation, Baltimore.*

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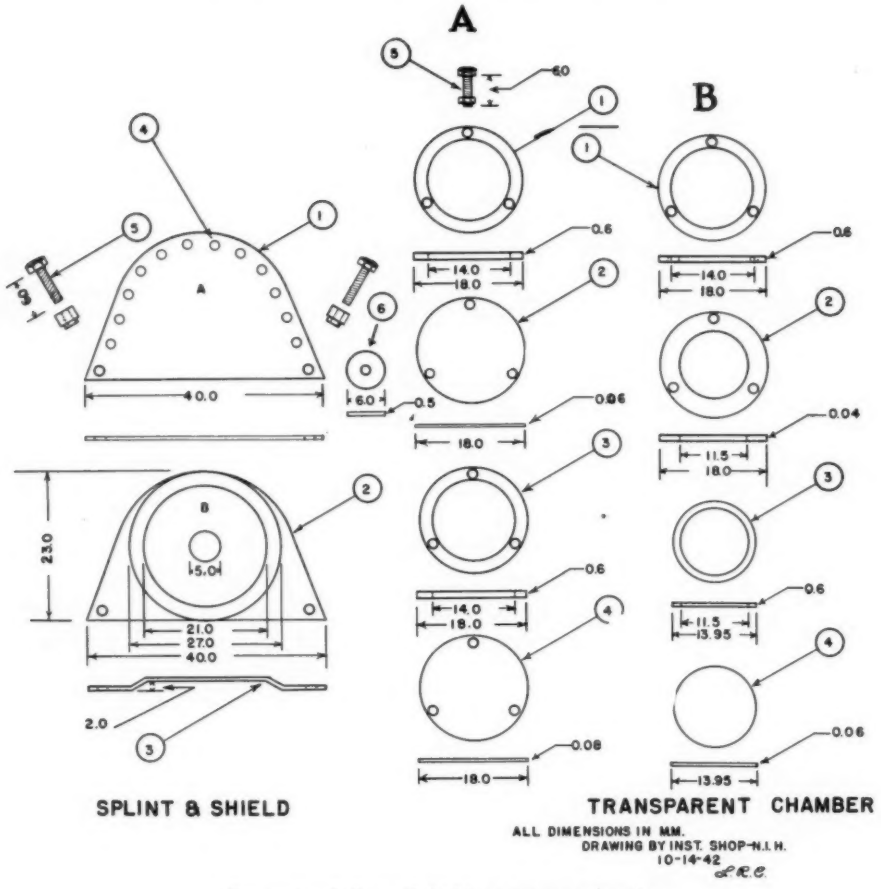

FIGURE 1.—Splint and shield; transparent chamber.

in the drawings, and each unit is presented in both the vertical and horizontal plane. The top piece consists of a ring of lucite, *1*, to which is cemented a cover slip of either mica or glass, *2*. The base, *3*, is constructed like the top and has a mica cover slip, *4*. Three silver bolts, *5*, are countersunk into the top lucite ring and cemented in position.[2]

Figure 1, *B*, shows the modification with a removable top cover slip which permits access to the living tissue. This modification, based upon the design by Williams (*6*) for the rabbit ear chamber, makes it possible to make implants of tumor cells or to carry out micromanipulative procedures at any time after the insertion of the chamber. The lucite top piece, *1*, is cemented to the mica ring, *2*. The retaining collar, *3*, to which is attached a thin glass cover slip, *4*, rests upon the inner rim of the mica ring. Collodion is used to seal it to the outer lucite ring. The retaining collar and cover slip may

[2] *The silver bolts were contributed by the Shuron Optical Co., Geneva, N. Y.*

be lifted out at any time for access to the tissue within the chamber.

SPLINTS AND SHIELDS

Experience of workers with the rabbit-ear chamber has shown the necessity of protective splints and shields in order to prevent trauma to the vascular tissue. In adapting the technique to the mouse, splints and shields (fig. 1) were designed not only to protect the chamber but also to give support to the double layer of skin and to maintain it in a vertical position after the insertion of the chamber. Both splints, *1*, and shields, *2*, are made of plastic sheet which can be cut to the desired shape with scissors or molded in a press to form a recess, *3*. A row of holes, *4*, around the periphery of the splint serves as the suture line in the production of the skin flap. Silver bolts, *5*, and rubber washers, *6*, are used for attachment of the splints and shields.

OPERATION

Skin Flap

The operation is carried out under urethane anesthesia and with strict aseptic precautions. The dorsal skin layer is clipped and cleaned with sterile green soap followed by metaphen 1:500 (aqueous solution). The mouse is placed on the rotatable stage of the dissecting microscope. At the center of the stage is an anvil of lucite (fig. 2, *1*, and *2*) which serves both as a base against which the holes for the bolts are punched and as a medium for transmitting light through the skin flap during the operation. The redundant skin is approximated along the line of the vertebral column, and two interrupted silk sutures are placed through this double skin layer, avoiding the large blood vessels. Two holes are punched for the bolts which are to support the splints: sites are selected, cephalad and caudad, respectively, to the vessels from the axillary and inguinal regions. A splint is then attached to the bolts, and the redundant skin provided by the procedure is sewed to the periphery of the splint by using a continuous suture. This provides a stretched double layer of skin of from 20 to 25 mm. diameter for the insertion of the chamber. Figure 3 shows the end result as well as the operative procedures by which the chamber is inserted into the skin flap.

Insertion of the Transparent Chamber

Three holes are punched which penetrate both the skin layers and the underlying splint. Then the holes in the splint are enlarged with a fine triangular file to permit free passage of the bolts. Under the dissecting microscope, radial incisions are made through the top skin layer, extending from each hole to the center. The three segments thus formed are reflected by means of small bulldog clamps. The superficial fascia is then carefully dissected away from the vascular bed of the underlying skin layer. The micro-implant of tumor cells is made at this time, unless the removable top chamber (fig. 1, *B*) is used. The bolts of the top piece of the chamber are pushed through the three previously prepared holes, and the top skin segments are drawn up about the outer rim. The vascular bed of the bottom skin layer is thus brought into direct contact with the underside of the cover slip.

In continuing the operation, the upper skin segments are trimmed to fit the outer edge of the chamber. Drying of the exudate attaches the skin to the chamber edge. In recently designed units this has been facilitated by having the mica cover slip (fig. 1, *2*) project 1 mm. beyond the outer edge of the lucite ring.

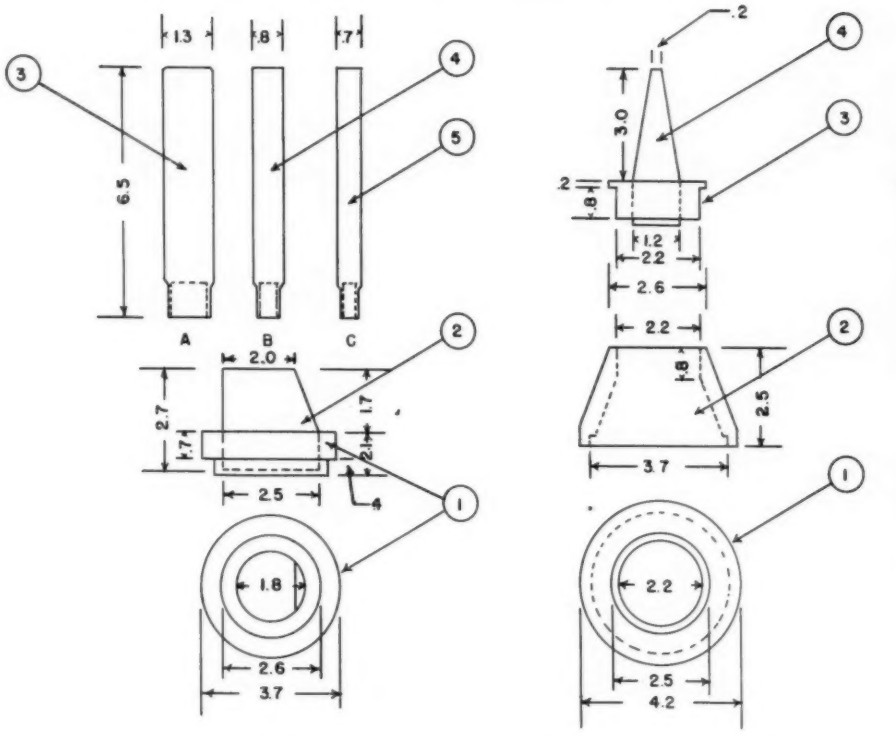

FIGURE 2.—Punches and anvil; quartz rod illuminator.

The base of the chamber is then slipped over the projecting bolts, and the nuts are screwed down, care being taken to avoid pressure on the tissue. It is possible also to omit the base and interpose plastic washers between the lower skin surface and the nuts.

APPARATUS

Figure 4 shows a general view of the apparatus.[3] The cast iron base supports the stereoscopic microscope, *1*, the compound microscope, *2*, camera, *3*, and drawing board, *4*. The carbon arc and tungsten ribbon filament lamps, *5* and *6*, are mounted on a sliding support which rests upon the shelf at the side of the base plate. The sliding support makes it possible to align either lamp with the substage mirror. Figure 6 shows in more detail the arrangement for rapid and accurate interchange of the stereoscopic and compound micro-

[3] *Laurence R. Crisp, Daniel P. Springston, and John F. Bronson, instrument shop, National Institute of Health, assisted in designing and constructing the apparatus.*

scopes. The complete stage and substage condenser assembly, *7*, were detached from the frame of the compound microscope [4] and mounted independently. The three guides, *8*, at the base make it possible to lock the frame of the microscope in position over the stage so that it is in optical alignment with the condenser. A turn of the knurled screw at the rear guide releases the microscope frame from its position. The mouse holder, *9*, transparent chamber stage, *10*, and straight quartz-rod illuminator, *11*, are described herein.

Mouse Holder

The mouse holder (figs. 5 and 6) was designed to immobilize the unanesthetized mouse. It consists of a brass tube, *1*, with a slotted groove, *2*, which supports the splint bolts as the mouse is guided into the tube. Air space is provided by holes at the tapered front end, *3*, as well as by the elongated openings, *4*. The plunger, *5*, serves to adapt the tube to the size of the animal and may be fixed in position by turning the lever, *6*. The plunger is cut away on one side to provide space for the tail.

Transparent Chamber Stage

The transparent chamber stage (figs. 5 and 6) supports the mouse holder and transparent chamber. The base, *1*, is mounted on the mechanical stage of the microscope. The guide, *2*, fits into the beveled slide of the mechanical stage and is made level by means of the spacer, *3*. A hole, *4*, in the base plate serves for the passage of light to the chamber. The mouse holder is then supported at correct level by means of the brass rod, *5*. Two pins in the base of this rod fit into the holes, *6*, of the base plate, and the transparent chamber support, *7*, slides into the guides, *8*. These are shown also in side view, *9*. The adapter, *10*, slips over the pins, *11*, which project through the holes, *12*, and is designed to receive the projecting chamber bolts in its slotted openings. The spring hold-down, *13*, holds the splint bolts firmly in position against the adjustable support, *14*. The spring clip, *15*, in contact with the margin of the transparent chamber holds it firmly in place. Figure 6 shows the mouse in the mouse holder with the transparent chamber in position for microscopic study.

FIGURE 3.—Mouse with transparent chamber in skin flap; melanoma implant beneath cover slip.

Transillumination

A tungsten ribbon filament lamp is used for visual observation at all magnifications. Photographic records may be made by using either this lamp or a carbon arc when exposures of a fraction of a second

[4] *Zeiss microscope stand FZE was used.*

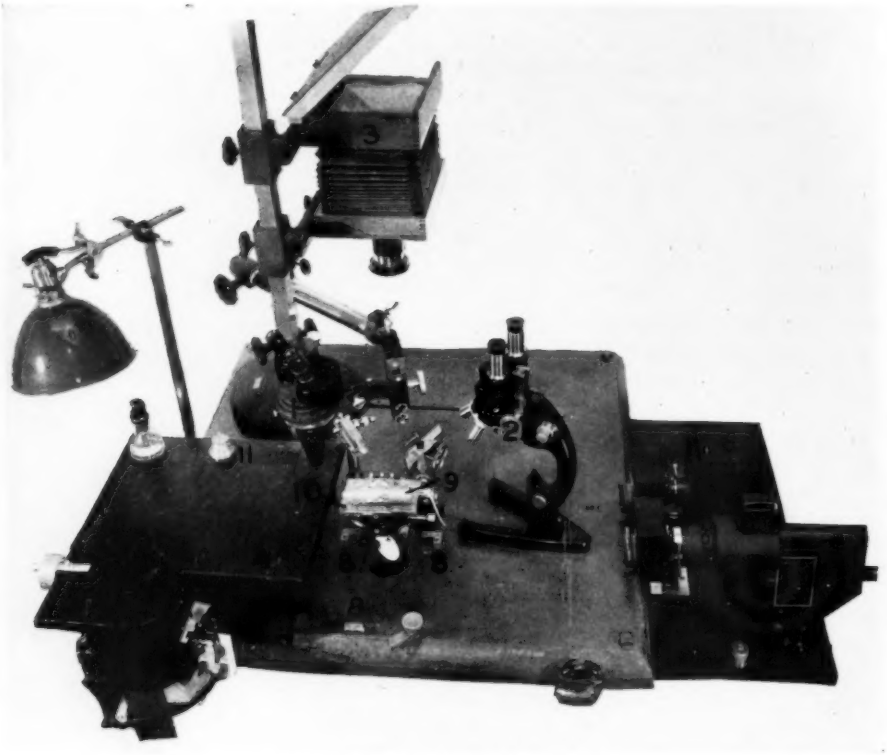

FIGURE 4.—General view of apparatus.

are desired. A photoelectric exposure meter arranged as described by Earle (*7*) is used to determine correct exposure time. Copper sulphate solutions and glass heat filters are used in the path of light from each lamp in order to prevent damage to the tissues through heating effects. The lower lens of the aplanatic condenser is used for low-power observation with the stereoscopic microscope or at magnifications up to 200 with the compound microscope. A straight quartz rod previously described (*8, 9*) is used to transmit light for observations at magnifications from 200 to 500 which is at the limit of effective resolution through relatively thick tissue. The quartz-rod illuminator (fig. 2) consists of a condenser mount, *1* and *2*, which fits over the lower lens of the substage condenser. An adapter, *3*, supports the quartz rod, *4*. The entire unit may be racked up and down with the substage condenser and thus brought into position directly beneath the basal skin layer of the transparent chamber.

TISSUE IN THE TRANSPARENT CHAMBER

Reference to figure 3 shows that the cover slip of the chamber is in contact with the inner surface of the bottom layer of skin. As the microscope is focused down through the chamber, one sees first the superficial fascia forming the floor. Under that may

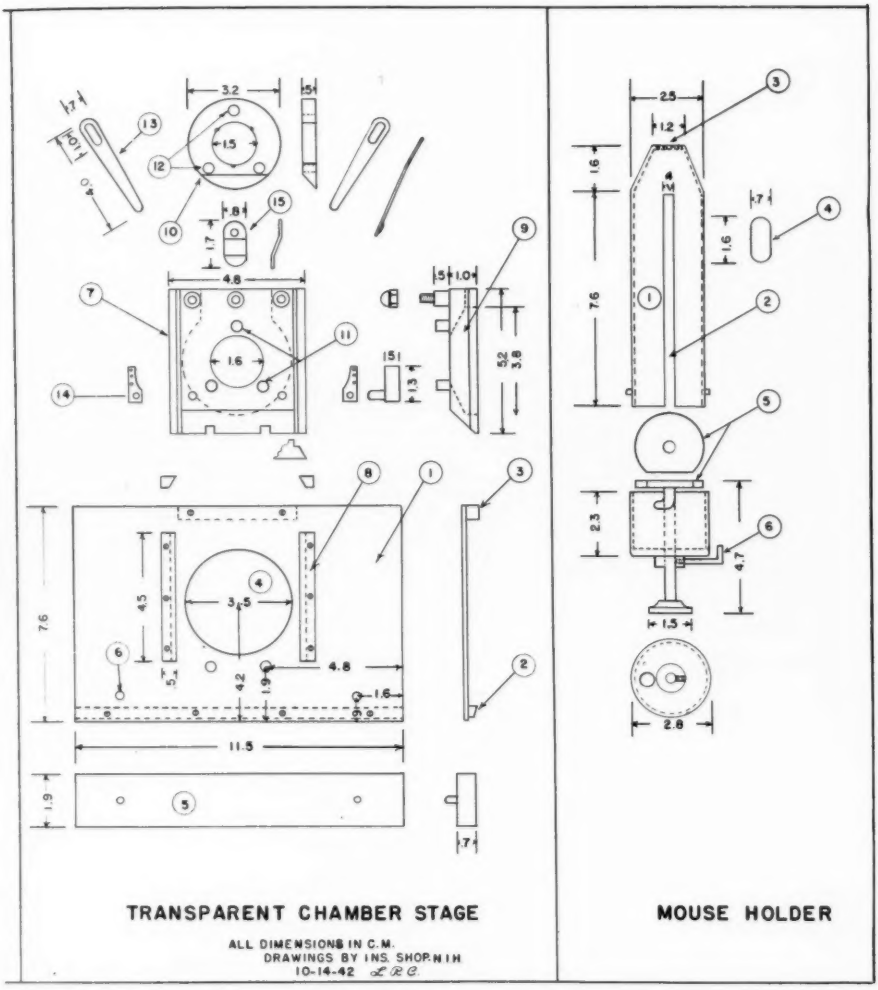

FIGURE 5.—Transparent chamber stage; mouse holder.

be seen the panniculus carnosus, and finally by focusing even lower, the subcutaneous connective tissue in which are found the fat cells and hair follicles. At low magnifications, as in figure 7, several tissue layers are seen in the same focus. This is a photograph of a melanoma implant upon the vascular bed. The tumor clump is surrounded by branches of the underlying main artery and vein, fat cells, and hair follicles.

The superficial fascia which forms the floor of the chamber is a layer of connective tissue with a relatively scanty blood supply. Two to three nerve trunks cross the chamber area at this level and are supplied by branches from the main vascular layer. Figure 8 shows a nerve trunk

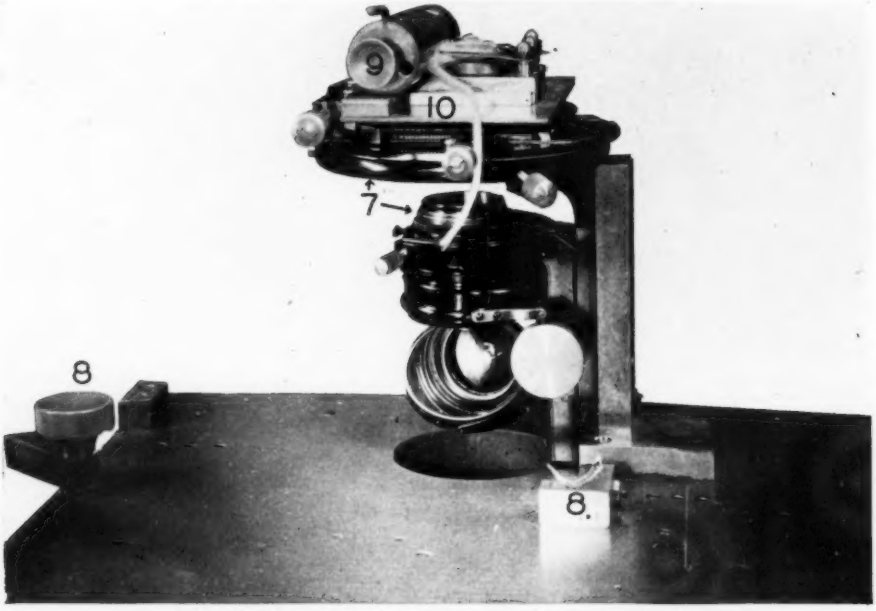

FIGURE 6.—Transparent chamber mounted upon mechanical stage.

FIGURE 7.—Melanoma implant on vascular bed of tissue within a transparent chamber, showing fat cells and underlying hair roots. × 20.

bordered by fat cells, with branches from the deeper vessels in the subcutaneous connective tissue. In instances where it is desired to place a tumor clump directly upon the extremely vascular layer of the panniculus carnosus muscle, the connective tissue layer usually is dissected away prior to the transfer. This leaves the panniculus carnosus as the floor of the chamber on which the tumor clump rests. This layer of striated muscle is supplied by parallel capillaries (fig. 9) which run between the muscle fibers and have their origin from branches of the main arteries and veins.

The subcutaneous connective tissue layer which lies beneath the muscle layer contains fat cells, hair follicles, and hair roots with the pigmented epithelial cells of the hair matrix. These are shown in figures 8, 9, and 10. The main artery and vein run through this layer, giving off branches to the panniculus carnosus and to the underlying dermal papillae and sebaceous glands. These two last-mentioned structures lie too deep for observation. As yet no reason has arisen for inserting a chamber using these lower layers.

FIGURE 8.—Nerve trunk in subcutaneous fascia, showing its vascular supply and neighboring fat cells; also trypan blue stained histiocytes in subcutaneous tissue. × 50.

DISCUSSION

More than 50 chambers have been inserted into mice in the course of the development of this technique. Up to the present it has been possible to maintain the vascular bed in good condition for from 30 to 40 days before sloughing occurs. Until this happens, microscopic examination of the blood vessels reveals a physiologically stable vascular bed. After the operation, no stasis or other evidence of damage to arteries, veins, or capillaries is found. A moderate hyperemia occurs within 24 hours after the operation but subsides within a day or two. In contrast with the preformed chamber in the ear of the rabbit, where from 7 to 12 days are required for recovery from the operation (*10*), the chamber in the mouse may be used immediately after its insertion. On the other hand, the cartilage of the rabbit ear affords greater stability, and the chambers last much longer in this site than in a skin flap in the mouse. It is of some importance that with the preformed type of chamber in the rabbit ear, observations are limited to relatively low powers of the microscope while in the same type of chamber in the mouse the range of effective magnification of the cells is from 15 to 500.

The technique described here has been used continuously since July 1942 and is now being used for making direct micro-

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FIGURE 9.—Parallel capillaries between muscle fibers of panniculus carnosus, showing underlying fatty layer and main vessels. × 100.

scopic studies of the early growth of transplantable tumors of the mouse. In addition to its usefulness in cancer research, the method has been found applicable to studies of tissue changes resulting from exposure to various physical and chemical agents.

SUMMARY

A method is described for making microscopic studies in vivo, of tumor growth in the mouse. In the development of the technique two types of transparent chambers were designed. These chambers are inserted into a skin flap of the mouse and are so constructed that micro implants of tumor cells can be made either at the time of operation or subsequently.

By the use of this technique it is possible to carry on prolonged microscopic observations of the living tissue (30–40 days) at magnifications up to 500.

In order to make possible direct microscopic study of tumor growth in the mouse, methods have been worked out and apparatus has been designed to immobilize the unanesthetized mouse for prolonged periods of time, to support the transparent chamber on the mechanical stage of the microscope, and to facilitate rapid interchange of the stereoscopic and compound microscopes in the examination of the living tissue.

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FIGURE 10.—Hair follicles and hair roots with pigmented epithelial cells of the hair matrix, also fat cells. × 280.

Microscopic Studies of the Early Growth of a Transplantable Melanoma of the Mouse, Using the Transparent-chamber Technique

By GLENN H. ALGIRE,[1] *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

In the previous article (*1*) a method is described for inserting a transparent chamber into a skin flap in the mouse for the purpose of making microscopic studies in vivo of tumor growth.

The present paper is an account of preliminary results obtained by the use of this technique in a study of the growth of the Cloudman melanoma (S 91) (*2*). These exploratory studies, carried out during the development of the technique, illustrate the application of the method to problems of cancer investigation.

Most microscopic studies of tumor growth have been made upon fixed, sectioned, and stained material. Such studies have, of necessity, been limited to a series of discontinuous stages in the growth of cells obtained from different animals killed at various intervals. This limitation makes it impossible to observe the continuity of changes undergone by any one selected group of cells and thus provides greater opportunity for error in interpretation. The cultivation and direct observation of tumor cells within the living animal overcomes this difficulty by making it possible to carry on daily examinations of the same group of cells over a prolonged period. Instances of the problems of cancer research which can be investigated in this way are (1) early stages in the growth of transplanted tumors, (2) the development of the vascular system, (3) the mechanism of tumor metastasis, and (4) the action of therapeutic agents on tumor cells.

Several recent workers attempted to solve the difficult technical problems involved in these studies in vivo. Lucké and Schlumberger (*3*) used the method of slit-lamp microscopy in studies of a carcinoma of the frog. By this method, observation and photography of living frog carcinoma could be made at magnifications up to 100. Other workers used the transparent-chamber technique for the study of normal living tissue in the ear of a rabbit, a method developed by Sandison (*4*, *5*), and later perfected by Clark, Kirby-Smith, Rex, and Williams (*6*). With this technique, using the preformed tissue type of transparent chamber, Ide, Baker, and Warren (*7*) observed the vascularization of the Brown-Pearce rabbit epithelioma. Their studies, also, were limited to the dissecting microscope with a range of magnification up to 100.

The mouse was selected for the present work because of the many inbred strains available, and because of the large number of tumors of this animal which have been carefully studied. In adapting the transparent chamber to the mouse, the objectives were (1) to maintain the tumor cells under normal environmental condi-

[1] *Fellow, Finney-Howell Research Foundation, Baltimore.*

tions of growth and nutrition, (2) to permit daily microscopic examination of tumor cells growing within the living mouse, and (3) to provide for the examination and photography of living cells at magnifications of the compound microscope up to 500.

MATERIAL AND METHODS

The methods employed in the present study have been described (*1*). Small fragments of the melanoma were placed upon the surface of the vascular bed either at the time of insertion of the chamber or a few hours afterward. The subsequent development of the transplants was observed through both the stereoscopic dissecting and the compound microscope at magnifications up to 120 with the former and 500 with the latter. Permanent records were obtained by photographs. Observations have been carried on in the same mouse for as long as 36 days, at the end of which time the tumor was fixed in Orth's fluid and stained with iron haematoxylin according to the method of Lillie and Earle (*8*).

GROWTH OF MELANOMA IN THE TRANSPARENT CHAMBER

Successful growth of melanoma tissue has been obtained so far in 5 of 10 attempted transfers. It has been possible to carry out microscopic studies on the tumor cells for periods of from 10 to 36 days. In some instances implantation of the tumor clump resulted in an excessive exudative reaction which obscured visibility and usually resulted in the failure of the cells to grow. In other cases exudative reaction was very slight, but the pigment of the melanoma cells was phagocytosed by host macrophages, and vascularization failed to occur. Figure 1 shows such a degenerating tumor clump at the end of 23 days after implan-

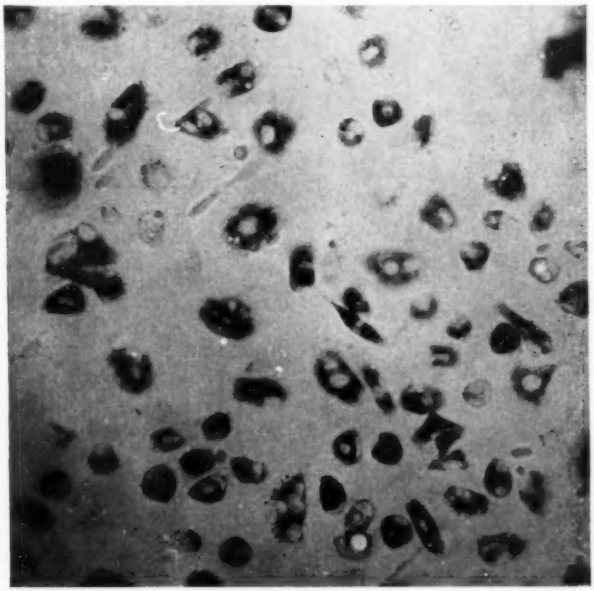

FIGURE 1.—Degenerating pigmented cells 23 days after implantation. × 280.

tation. The cells contain droplets and clumps of melanin pigment.

None of the implants that took showed subsequent regression. In the successful cases, the tumor cells eventua'ly grew beyond the confines of the chamber. Usually the tumor clump was obtained from a subcutaneous transplant. However, in the one case in which the growth could be followed for 36 days, the cell clump was obtained from a metastatic melanotic nodule in the lung of a strain dba mouse bearing the tumor. The description which follows is based largely upon observations made in this animal.

The operation for the insertion of a chamber of the removable-top type was carried out in the usual way (*1*). The host mouse bearing the melanoma was killed, the lungs were exposed, and a minute, pigmented, metastatic nodule was removed to a sterile culture slide. Under

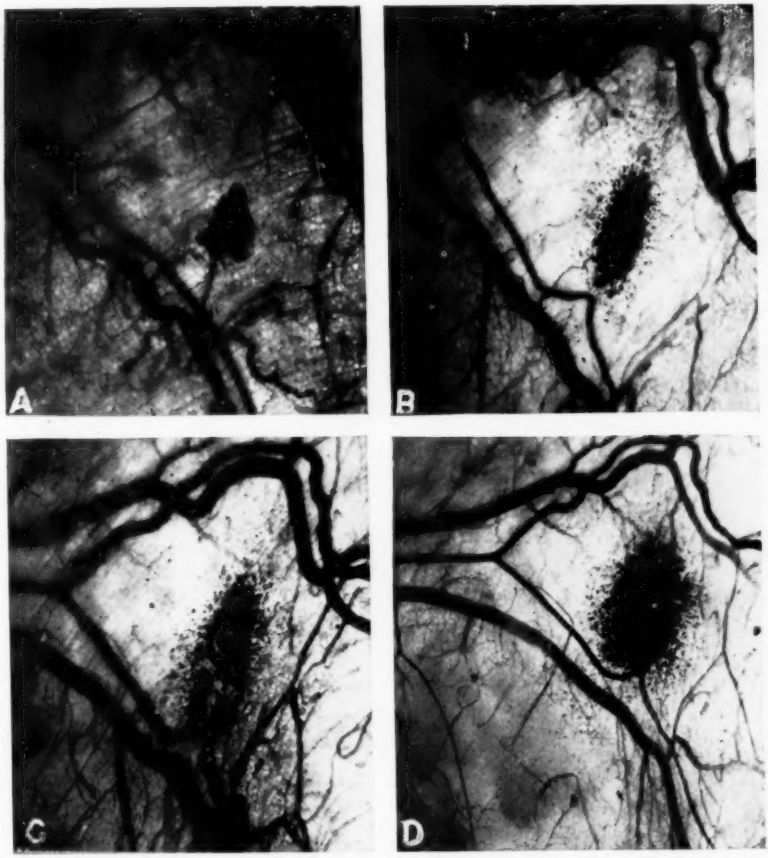

FIGURE 2.—Melanoma growth on vascular bed of tissue within a transparent chamber. × 15. *A*, Immediately after transfer of tumor tissue; *B*, The melanoma at the time of beginning vascularization; *C*, Four days after vascularization was observed; and *D*, Enlargement of vascular branches to the tumor.

the dissecting microscope, a small fragment of the tumor clump was teased away from the main mass of tissue with microneedles. The cover slip of the chamber was then removed and the tumor clump placed on the surface of the vascular bed, after which a new sterile cover slip was placed in position.

Figure 2 is a group of low-power photographs showing the development of the tumor clump over a period of 28 days. Figure 2, *A*, was taken on the day of transplantation, *B*, on the twentieth, *C*, on the twenty-fourth, and *D*, on the twenty-eighth. Figure 2, *A*, shows that the recently implanted pigmented tumor clump rests upon the subcutaneous fascia overlying the panniculus carnosus. The muscle capillaries are visible as parallel lines in the photograph. The clump at this time appeared rounded up, and its dark brown pigment contrasted sharply with the surrounding vascular tissue. On the following day, the tumor tissue was found to be attached to the underside of the cover slip rather than to the vascular bed and was surrounded by a local accumulation of fluid, in which were observed leukocytes and macrophages. During the next few days the melanoma became surrounded by numerous macrophages containing ingested melanin pigment.

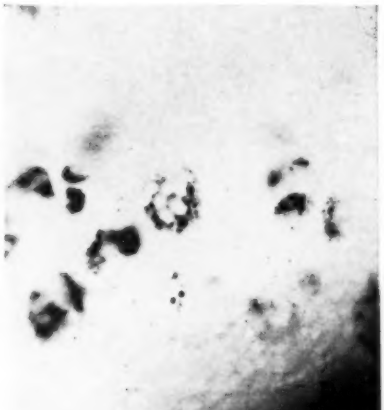

FIGURE 3.—Phagocytes and multinucleated cells containing clumps of pigment. × 280.

Up to the tenth day after implantation there was very little enlargement of the tumor clump. The tumor cells remained rounded, and no cell processes were observed. The cells were oval and contained relatively large ovoid nuclei with one to two nucleoli. The cytoplasm contained small pigment granules.

Between the tenth and twelfth days, multinucleated cells containing 3 to 5 nuclei appeared on the under surface of the cover glass in the region surrounding the melanoma. Some of these developed from 12 to 40 nuclei during the next few days. Their cytoplasm contained large, irregular clumps of brown pigment, indistinguishable from those in the host macrophages. These are shown in figure 3.

On the fifteenth day the tumor clump was found to be attached to the vascular bed. By the seventeenth day, cellular migration had increased, and tumor cells with pigmented processes were observed to have migrated from the edge of the tumor (fig. 4). These differed from the host macrophages and giant cells in that their cytoplasmic pigment granules were uniformly small. The uniformity in size of the granules is shown also in figure 6. It was observed that the most distally located cells contained fewer granules than those close to the original clump.

Beginning vascularization of the tumor clump was observed on the twentieth day after implantation, when new capillary tips were observed to have grown into the tumor from an adjacent venule. Figure 5 shows the tip of a newly formed capillary, the lumen of which contained erythrocytes and leukocytes. The new capillaries were dilated with blood cells which pulsated to and fro with the pres-

FIGURE 4.—Tumor cells with cytoplasmic processes containing pigment granules of uniform size. × 280.

sure from the vessel of their origin. The growing endothelial tip was narrowed to a point. Figure 2, *B*, shows the size of the tumor clump 20 days after implantation, at which time vascularization was first observed. Following this, the growth of the tumor became greatly accelerated, as is seen by comparing the photographs in figure 2. Capillaries were observed to grow into the tumor mass very rapidly during this period, and by the twenty-eighth day the vascular bed was well-established. Figure 2, *D*, shows the size of the cell clump at this time, as well as the enlargement of the vascular branches from which the tumor had received its new vessels. No new capillary tips were observed after the twenty-eighth day, and the vessels within the tumor had lost the dilated, engorged state characteristic of newly formed capillaries. Figure 6 shows the growing edge of the melanoma with well-developed functional capillaries, between which lie the tumor cells.

The photograph of the entire tumor mass taken on the thirtieth day (fig. 7) shows a rapidly growing tumor with radially migrating cells and an established vascular supply. The transparent chamber was removed from the mouse on the thirty-sixth day, and the tumor was fixed in Orth's fluid as a whole mount.

DISCUSSION

While the experiments were only tentative and exploratory, certain facts appear to warrant emphasis.

It is of interest that such a long interval as 20 days elapsed between the implanta-

FIGURE 5.—Newly formed capillaries within tumor. × 280.

FIGURE 6.—Tumor cells at the margin of the melanoma, and the stable vascular bed. × 400.

tion of the tumor and the beginning of vascularization. In this connection, it is important to point out that the cell clump was placed upon an uninjured vascular bed, rather than being associated with the production of a wound as is the case when a tumor fragment is implanted with a trocar. Jones and Rous (*9*) attributed the secondary localization of tumors at points of injury to the presence at such points of an active connective tissue capable of elaborating a stroma rapidly and abundantly. Unpublished observations of the writer on the healing of wounds in transparent chambers in the mouse show that new capillaries begin to grow at approximately the seventh day after injury. Further work will be necessary to determine the relation between tissue injury and the vascularization of tumor implants.

The vascular pattern of this melanoma corresponds to that found by Lewis (*10*) for the Walker fibrosarcoma, with large afferent and efferent vessels and a rich capillary plexus.

Only one type of tumor cell was found in the melanoma. The cell could be readily distinguished from the tissue macrophages by the presence of numerous pigmented intracytoplasmic granules of uniform size.

The multinucleated cells which were observed near the tumor implant between the tenth and twelfth days appeared to have developed from the host macrophages surrounding the tumor. They were found also about tumor clumps which failed to grow. They were not derived from the tumor cells. These cells remained attached to the underside of the cover slip and did not migrate, and most of them gradually disappeared during the succeeding period of study.

This melanoma readily metastasizes to the lungs of strain dba mice. In the

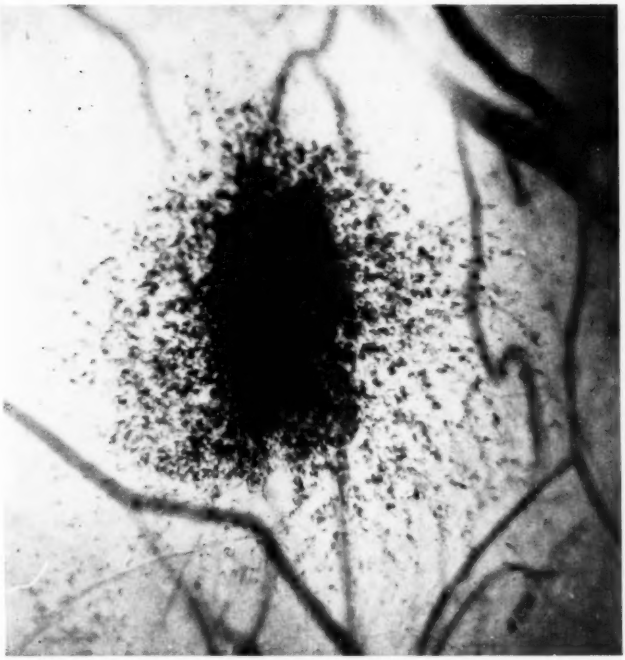

FIGURE 7.—Radial migration of tumor cells from the melanoma. × 45.

animal in which the transplant was followed in the transparent chamber 36 days, no tumor cell emboli or secondary metastatic growths were observed. Although the animal was kept alive 3 months after the chamber containing the tumor had been removed, no lung metastases were found at necropsy.

The preliminary results recorded here show the usefulness of this method of studying tumor growth in vivo. It remains for further work to extend these observations.

SUMMARY

Melanoma cells were planted on uninjured vascular tissue contained within a transparent chamber inserted into a skin flap in the mouse. Daily microscopic examinations were made of tumor cells growing under normal environmental conditions, for periods up to 36 days.

Vascularization began after an interval of 20 days. During this interval there was little growth of the tumor. Prior to vascularization the tumor clump was surrounded by leukocytes, macrophages, and multinucleated cells, which appeared in the order given. The multinucleated cells were not derived from the tumor cells, but from the macrophages. After vascularization, migration and proliferation of the tumor cells became accelerated.

The melanoma cells were readily distinguishable from pigment-containing macrophages by the greater uniformity of size of their pigment granules.

The vascular bed was stabilized in the tumor 8 days after its inception. No tumor emboli or metastases were seen.

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Estimation of Growth Rates of Tumors

By HAROLD F. BLUM, *senior pharmacologist*,[1] *National Cancer Institute, National Institute of Health, United States Public Health Service*

Rate of growth of tumors is an important index which may be employed in cancer studies of various kinds. However, measurements of growth are subject to considerable error both in experimental determination and in interpretation. There seems to be no generally accepted method either for obtaining or reporting tumor growth rates, and hence the following discussion seems in order. The basic concept is not novel, being the principle of exponential growth or compound interest. It has been applied to growth processes in general and is fundamental thereto. (See Fisher (*1*), and Shock and Morales (*2*).)

Since the growth of a tumor depends basically upon proliferation of tumor cells, the fundamental equation for exponential growth applies. This may be written as follows:

$$\log \frac{N_2}{N_1} = \mathrm{K}(t_2 - t_1) \qquad (1)$$

where N_1 is the number of cells at time t_1, N_2 the number of cells at time t_2, and K a constant. This equation merely states that the number of cells produced within a given time is proportional to the total number of cells present. For numerous reasons, this simple equation cannot be expected to hold from beginning to end of the tumor growth process, but its implications cannot be escaped, and it is safe to assume that it holds for any short interval of time.

If the tumor may be assumed to be homogeneous, an assumption which may also be expected to hold at least for short intervals of time, the volume, V, is proportional to the number of cells, and:

$$\log \frac{V_2}{V_1} = K(t_2 - t_1) \qquad (2)$$

where V_1 is the volume at time t_1, V_2 the volume at time t_2 and K the appropriate constant. Under the conditions stated, K is numerically equal to K.[2] Equation (2) may be rearranged in convenient form for calculating K, which is the proper index of tumor growth to be used for comparative studies:

$$\frac{\log V_2 - \log V_1}{t_2 - t_1} = K \qquad (3)$$

To illustrate the relationship to cell division: when $\log V_2 - \log V_1 = \log 2$, a doubling of the initial volume is indicated, and $t_2 - t_1$ represents the average time required for a complete cell division.

Thus, if the volume of a tumor could be accurately measured at different times, the constant K could be employed as a proper index of the rate of tumor cell proliferation, at least over short intervals of time. However, it is seldom feasible to measure tumor volumes, and one is usually limited to linear measurements of tumor diameters. Sometimes the three major axes of the tumor may be measured; more often only two or possibly only one diameter can be determined; but assuming that such measurements may be obtained with sufficient accuracy, it should be possible to estimate tumor growth from them in certain cases at least, provided attention is given to the geometry of the problem.

Measurements of diameters can be strictly reliable for estimation of tumor growth only in the case of tumors which change their volume without changing

[1] *Now principal biologist, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md.*

[2] *The same is true if tumor weights are substituted for tumor volumes, and hence the same method of comparison should be used when this measure can be employed.*

their shape. This limits accurate estimations to "smooth" tumors, that is, tumors which closely approach a simple geometrical form, and those which do not change that form appreciably during the course of the measurements. This means that accurate estimates of tumor growth are to be obtained only on selected tumors or tumor types. Actually, as will be pointed out later, it may be possible to make fairly reliable estimates for somewhat irregular tumors if proper judgment is exercised by the observer. However, as the limits of accuracy cannot be directly determined, such estimates should be viewed with a modicum of distrust.

Probably the most common form of measurable tumor is spheroidal in shape. The volume of a sphere or spheroid may be expressed:

$$V=4/3\pi\frac{a}{2}\times\frac{b}{2}\times\frac{c}{2} \qquad (4)$$

where a, b, and c are the three diameters or dimensions along the three major axes of the spheroid. In a spheroid, either oblate or prolate, two of the diameters, a, b, c, are equal; in a sphere all three are equal. Where it is possible to measure all three diameters, the volumes may be calculated and substituted in equation (3) to obtain a value for the constant K. The calculation may be simplified, since it is unnecessary to determine the individual volumes. From (3) and (4) we obtain

$$\frac{\log 1/6(\pi a_2b_2c_2)-\log 1/6(\pi a_1b_1c_1)}{t_2-t_1}=K \qquad (5)$$

and canceling out:

$$\frac{\log a_2b_2c_2-\log a_1b_1c_1}{t_2-t_1}=K \qquad (6)$$

If it were possible to measure only one diameter of such a tumor, the rate of growth might still be estimated. If the tumor does not change its geometrical shape as it grows, the dimensions a, b, and c, maintain proportionality. Thus, if k' and k'' are proportionality constants,

$$b=ak' \qquad (7)$$

$$c=ak'' \qquad (8)$$

and

$$V=\frac{1}{6}\left(\pi a^3k'k''\right) \qquad (9)$$

If a_1 is one diameter measured at time t_1 and a_2 this diameter at time t_2, the following equation is obtained from (3) and (9):

$$\frac{\log 1/6(\pi a_2^3k'k'')-\log 1/6(\pi a_1^3k'k'')}{t_2-t_1}=K \qquad (10)$$

Canceling out:

$$3\left(\frac{\log a_2-\log a_1}{t_2-t_1}\right)=K \qquad (11)$$

A spheroidal tumor may have its profile exposed, permitting the measurement of two diameters a and b, but not the third. By reasoning similar to the above, it may be shown that

$$\frac{3}{2}\left(\frac{\log a_2b_2-\log a_1b_1}{t_2-t_1}\right)=K \qquad (12)$$

If the tumors were exact spheroids, accurate measurements of one diameter would be sufficient if used with the proper equation (11). However, the accuracy of such measurements is always limited, and the averaging of three measurements which occurs when using equation (6) should give a more reliable value of K, provided the measurements are equally accurate However, it may be obvious at times that measurement of one diameter is more reliable than that of another. For example, in the case of a very flat, oblate spheroid, measurement of the smallest diameter may be less accurate than measurement of the other two. In such a case, only the two best diameters should be used and K calculated from equation (12). In some cases the use of only one diameter

and equation (11) may be advisable. The observer can determine the better procedure in such cases.

Sometimes growth may be restricted at one plane, so that the tumor assumes a hemispherical shape. In such cases the same equations, (6), (11), and (12), should apply, since if growth is proportionate in all dimensions the half diameter perpendicular to the base increases only half as fast as it would if the tumor were a complete sphere. This has the effect of dividing the value of, say, k' in equations (7) and (10) by 2, and does not change the value of K. The same would be true if the tumor were some fraction of a sphere other than half, provided the difference in volume were caused by flattening along a single plane, and the same would be true of a spheroid flattened perpendicular to one of its major axes. It is this type of flattening which is to be expected under the conditions that determine tumor growth.

The only requirement for the application of such measurements is that the tumor maintain the same shape as it grows. As a rule this is only true for relatively short periods of growth, as the "smoothest" tumor sooner or later becomes irregular. For short periods of time it is probable that even tumors of irregular shape approximate the condition of proportionate growth along all dimensions and that fair estimates of growth during short periods may be made on such tumors. When this is attempted, it would seem best to select a single dimension which can be most accurately measured and apply equation (11). But here again the observer must exercise judgment, for there is no way to estimate directly the reliability of measurements so obtained.

There are other considerations which make it advisable to limit the measurement of tumor growth for comparative purposes to relatively short intervals. The value of K is an index of growth by cell proliferation at any period of time during which other conditions that affect tumor growth remain constant. Such conditions do not remain constant throughout the growth of the tumor, and it is probable that they influence the rate of cell profiferation as well as other factors. For example, the blood supply to the tumor changes as the tumor grows. Exponential growth cannot continue without limit, and tumors grow more slowly at late stages of their development. Studies by the writer (*3*) also suggest that growth is slow during the early stages of tumor development. It is highly improbable that tumor rates ever remain constant throughout the course of tumor development, and hence K can be assumed to be constant only for limited periods. Obviously, then, it is dangerous to compare the growth of very small tumors with very large tumors, unless it is definitely known that K remains constant throughout the range of tumor sizes under consideration for all tumors of the type under study. If comparison is to be made between tumors of the same type in the same species of animals, K should be a good index of comparison if it is determined for tumors of approximately the same size. But comparison of different tumor types, and particularly of tumors in different species of animals must rely upon more or less arbitrary assumptions and great reliability should not be ascribed to such comparisons. Nevertheless, relative growth rates, which are measured by K, provide the only significant means of comparison.

Another geometrical form is of interest, a flat cylinder of a constant height. It is improbable that this form is found frequently in tumors. However, it is the form which tissue cultures growing on the surface of a solid medium may be expected to take and hence is of interest in cancer research. The volume of a

cylinder of circular or elliptical base may be expressed as:

$$V=\pi\frac{a}{2}\times\frac{b}{2}\times h \qquad (13)$$

where a and b are the diameters of the base, and h is the height of the cylinder. Volumes may be calculated and used in equation (3), but usually it will be difficult to measure h, and K may be calculated from values of the two diameters a and b, or from only one of these. Following the same reasoning as above, the equation when both diameters are used is:

$$\frac{\log a_2b_2-\log a_1b_1}{t_2-t_1}=K \qquad (14)$$

or when only one diameter is used:

$$2\left(\frac{\log a_2-\log a_1}{t_2-t_1}\right)=K \qquad (15)$$

If it is desired only to make comparison of rates of tumor growth, certain short cuts in calculation are possible. If the same dimension or dimensions have been used throughout, it is unnecessary to calculate the values of K for comparison of the growth rates of different tumors. This is true since equations (6), (11), (12), (14), and (15) differ only in the number of measured diameters employed and in the value of a constant by which the left hand member of the equation is multiplied. If the latter constants are neglected we may write:

$$\frac{\log D_2-\log D_1}{t_2-t_1}=K' \qquad (16)$$

where D is the diameter (or product of two or three diameters) to be employed and K' is a constant which may be used for comparison of rates under the limited condition that the same number of diameters is used throughout. Under these conditions ratios of K' will have the same values as ratios of K. It is only necessary to calculate true values of K when it is desired to compare tumors for which different numbers of diameters have been measured.

In practice measurements will usually be obtained several times in the course of a tumor's growth, and K' may be best obtained graphically in this case. If the logarithms of the various values of D are plotted against corresponding values of t, the slope of the line is K'. This is quickly accomplished by using semi-logarithmic paper and drawing the best line through the points, which will usually be found to scatter considerably because of the inherent inaccuracies of such measurements. Where tumor growth rates are to be compared under different experimental conditions, more reliable comparisons may be obtained by averaging the values of K' for a number of tumors developing under each experimental condition. In the writer's experience the range of growth rates of tumors of the same type varies widely among individuals (*3*), and rather large samples may be necessary for accurate comparison. It is also necessary that the tumors be selected at random so that the sample includes both early and late appearing tumors.

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Morphology and Growth of Subcutaneous Tumors Induced with Carcinogenic Hydrocarbons in Strain C3H Male Mice

By MICHAEL B. SHIMKIN, *past assistant surgeon*, and W. RAY BRYAN, *biologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

The purposes of this investigation were to study the growth rate of tumors induced in strain C3H male mice by the subcutaneous administration of carcinogenic hydrocarbons, to describe the morphologic features of these neoplasms, and to determine the relations between the growth rate, latent time, the dose and type of carcinogen, and the histologic appearance of the tumors.

EXPERIMENTAL PROCEDURE

The material is provided from an experiment on the response of young C3H male mice to 12 logarithmically spaced doses of 20-methylcholanthrene (1.0 to 0.00024 mg.), 3, 4-benzpyrene and 1, 2, 5, 6-dibenzanthracene (8.0 to 0.00195 mg.) dissolved in 0.25 cc. of tricaprylin and administered as single subcutaneous injections.[1] The details concerning the grouping of the animals, the preparation and the dosages of the three hydrocarbon solutions, etc., are presented in the original communication (*1*).[2]

The mice injected with the three hydrocarbons were examined every 4 to 5 days. The latent times were estimated in terms of the interval between the injection of the hydrocarbon and the original diagnosis of tumor by observation and palpation. The tumor-bearing mice were then separated, and the tumors were measured every other day with a pair of calipers. Measurements were made of the two greatest diameters to the nearest millimeter, and approximately constant pressure was used.

Growth rate was expressed in terms of a constant, K, which represents the proportionate increase in tumor volume per unit of time. The values of K were derived by the use of equation 12 discussed by Blum (*2*) and reproduced as follows:

$$\frac{3}{2}\left(\frac{\log\ (a_2\times b_2)-\log\ (a_1\times b_1)}{t_2-t_1}\right)=K$$

where a and b represent the two diameters determined at a given measurement, and t represents the time at which the measurements were made. The subscripts 1 and 2 represent initial and final observations, respectively (or any two sets of measurements taken at different times). In the present study the diameters were expressed in millimeters and the time in weeks. K is therefore the logarithm of the proportionate increase in tumor volume per week. Thus, a K value of 0.301 would indicate a doubling of the tumor mass each 7 days. Since the earlier measurements, taken when the tumors were small, are subject to greater relative errors than the later measurements of larger tumors, two points on the smoothed growth curve for a given tumor were employed for the calculation of K, rather than actual initial and final measurements. For this pur-

[1] *Referred to as methylcholanthrene, benzpyrene, and dibenzanthracene hereinafter.*

[2] *Attention is directed to the following errata which appeared in the original communication* (1): *p. 514, the black dot on the base line between dose 0 and −0.6 is an imperfection of printing and not a plotted point; p. 524, footnote 11, the molecular weight of dibenzanthracene should be 278 and not 268; p. 530, second column, seventh line from bottom of page, 08.75 should read 0.875.*

pose, the diameter products were plotted on semilogarithmic paper against time. The majority of the plots gave an excellent fit to a straight line, but others followed a steplike course. In the latter event a straight line was drawn to represent an over-all average rate of increase, and points on this line were used in determining K. Most of the tumors were followed until they reached a size of approximately 20 by 20 mm., but some of the mice died before this size was reached. Tumors for which there were fewer than four measurements extending over a period of 1 week were not included in the analyses.

At the termination of the observation on growth, or upon the death of the animals, the local tumors were removed, fixed in Zenker's fluid, and embedded in paraffin. At least two, and in the majority of cases three sections were made through the tumor. The sections were stained with haematoxylin and eosin, and approximately half of the sections were also stained with Mallory's phosphotungstic acid-haematoxylin. No sections were

TABLE 1.—*Summary of data obtained with 3 carcinogenic hydrocarbons in male mice of C3H strain*

Dose		Mice with tumors (total)	Mean latent period	Tumors measured for growth	Mean rate of growth (K) [1]	Mice with multiple tumors	Tumors examined histologically [2]	Type of tumor		
								Sarcoma	Sarcoma with muscle-cell elements	Carcinoma [3]
Milligrams	*Log milligrams*	*Number*	*Months (days/30)*	*Number*		*Number*	*Number*	*Number*	*Number*	*Number*
20—METHYLCHOLANTHRENE										
1.0	0	20	2.40	20	0.52	2	19	8	10	1
0.5	−0.301	21	2.57	19	.47		21	10	9	2
0.25	−.602	21	2.77	20	.40	3	21	11	10	
0.125	−.903	21	3.32	20	.47	1	21	15	6	
0.0625	−1.204	17	3.92	16	.33	2	17	10	7	
0.0312	−1.505	13	5.24	12	.34		12	12		
0.0153	−1.806	6	4.59	6	.29		6	3	3	
0.00781	−2.107	3	6.97	3	.36		3	3		
3, 4—BENZPYRENE										
8.0	+0.903	20	2.96	17	0.49	1	18	7	11	
4.0	+.602	16	3.02	15	.45		14	7	7	
2.0	+.301	19	3.09	19	.43	3	19	11	7	1
1.0	0	18	3.32	18	.41	2	18	10	8	
0.5	−.301	19	3.86	17	.41	1	19	17	2	
0.25	−.602	14	4.41	13	.40		13	8	5	
0.125	−.903	15	5.11	15	.41	1	14	12	2	
0.0625	−1.204	4	5.79	4	.37		4	3	1	
0.00195	−2.709	2	8.37	2	.45		2	1	1	
1, 2, 5, 6—DIBENZANTHRACENE										
8.0	+0.903	16	3.75	12	0.32	1	16	11	5	
4.0	+.602	17	3.83	15	.36		17	9	8	
2.0	+.301	19	3.69	18	.38		19	11	8	
1.0	0	22	3.60	18	.48		21	11	10	
0.5	−.301	20	3.76	17	.42	1	19	11	8	
0.25	−.602	19	4.01	18	.47	2	19	16	3	
0.125	−.903	21	4.47	20	.35	2	20	16	4	
0.0625	−1.204	20	5.10	19	.39		17	16	1	
0.0312	−1.505	16	6.31	13	.33		13	12	1	
0.0156	−1.805	6	5.94	4	.44		6	3	3	
0.0078	−2.107	6	8.78	6	.38		5	3	2	
0.00195	−2.709	2	9.48	2	.44		2	2		
Total		433		398		22	415	269	142	4

[1] See text.
[2] Only the first appearing tumors of mice with multiple tumors are included.
[3] With or without sarcoma.

made of other tissues except when indicated by the presence of gross abnormalities.

In the original study, 957 mice were used, and 433 mice that developed progressively growing subcutaneous masses were included in the dose-response analysis. Of the tumors from these 433 mice, 415 were examined and verified histologically. Eighteen mice, on which histologic sections were not available because the tumor was devoured by other animals or was extremely autolyzed after the death of the mouse, were included on the basis of rapid growth of the local tumor.

For the present study, therefore, 415 mice with tumors are included in the morphologic observations; and 398 mice with tumors large enough and of which measurements were made, are included in the analysis of tumor growth. The mice were distributed throughout the dose and hydrocarbon groups included in the dose-response analysis. The number of mice with tumors and the growth data for each group are given in table 1. Twenty-two of the four hundred and fifteen animals with tumors had more than one distinct tumor; a separate analysis of these animals is given subsequently. Only the tumor that appeared first was considered in the data on latent time and growth rate.

MORPHOLOGY

Grossly, the tumors were spheroidal, fairly well circumscribed, subcutaneous masses, of firm to fairly soft consistence. They were usually fixed to the overlying skin, which in the case of the larger tumors often was externally ulcerated. On cut section, the masses were pearl-gray in color, areas of hemorrhage and necrosis being frequently encountered in large tumors. The tumors compressed the thoracic wall and were adherent to the ribs, the humerus, and other adjacent structures. Actual penetration into the thoracic or abdominal cavities was rarely encountered. Grossly, distant metastases were not observed. The regional lymph nodes were usually encompassed and invaded by the tumor, and in one case pulmonary metastases were found upon histologic examination.

The tumors grew progressively, and the cause of death in the majority of animals was attributable to the bulk of the tumor and secondary infection owing to ulceration.

Histologically,[3] the tumors were of the following types: sarcoma, 411, divided into 2 groups; spindle-cell sarcoma 269, and spindle-cell sarcoma with muscle elements 142; carcinoma, 2; and mixed sarcoma and carcinoma, 2.

Sarcomas

The sarcomas, which comprised 99 percent of the tumors, were in general spindle-celled growths, the cells varying widely within most of the neoplasms. In many instances the cells were arranged in interlacing bundles or broad sheets. Other tumors or other regions of the same tumor contained shorter and broader cells mixed with round or irregular forms and showed no characteristic structural arrangement.

The cells resembled fibroblasts. They were large, elongated, with acidophilic cytoplasm, and hyperchromatic, large, oval, vesicular nuclei, and prominent nucleoli. Fine fibroglia were observed in a considerable number of sections stained with phosphotungstic acid-haematoxylin. In more pleomorphic neoplasms fibroglia could not be demonstrated. Mitoses and giant multinucleated cells were abundant in almost all of the tumors. In some tumors the blood channels were extremely

[3] *Numerous illustrations of these tumors are presented by Bonser and Orr* (3), *Haagensen and Krehbiel* (4), *Lewis* (5), *and others, and reduplication here is considered superfluous.*

prominent and suggested a hemangiomatous structure.

Necrotic, hemorrhagic, and edematous areas of various sizes were present in almost all tumors that reached 20 by 20 mm. in diameter. Some tumors contained areas of hyaline and of myxomatous degeneration. In a considerable number of tumors appearing soon after the injection of the carcinogens were round cysts lined with compressed spindle cells, probably representing localized deposits of the injected material. Invasion by round cells and polymorphonuclear leukocytes occurred to a noteworthy degree only in tumors in which surface ulceration had occurred.

The tumors were not encapsulated, and they actively invaded and destroyed contiguous structures, such as muscle, lymph nodes, ribs, and long bones. The majority of the neoplasms were fibrosarcomas arising from the fibroblasts of the subcutaneous connective tissue. Bonser and Orr (*3*) suggested that some of the tumors may arise from the endomysium of the panniculus carnosus or of the deeper muscles. The structural variability of the individual tumors suggests that several types of cell may undergo malignant transformation, although from morphologic evidence alone it is possible that the variants are the result of accidental differences in location, pressure, and other factors that produce alterations in appearance of essentially similar cells.

Sarcomas With Muscle Elements

About three-fourths of all 411 sarcomas contained occasional large eosinophilic cells with multiple nuclei, which varied in size, length, and number of nuclei. The cells were modified muscle fibers or muscle giant cells. In 142 tumors (34.5 percent) these muscle elements were sufficiently numerous and prominent to deserve separate consideration. All these tumors contained spindle cells that were intimately associated with the muscle elements, and many also contained other areas of sarcoma that were free of muscle elements. The muscle elements usually occupied the deeper portion of the tumor and in several cases could not be certainly distinguished on morphologic grounds from rhabdomyosarcomas, although no cross striations could be demonstrated.

Abnormal muscle elements in tumors induced by carcinogenic hydrocarbons in mice have attracted comment in all descriptions in the literature (*3–6*). The nature of these cells is not clear. Haagensen and Krehbiel (*4*) and Hval (*6*) considered 3 of 32 tumors studied by them as rhabdomyosarcomas or leiomyosarcomas. Bonser and Orr (*3*) and Lewis (*5*), on the other hand, considered them as a reaction of injury to muscle produced by the hydrocarbon. It cannot be denied that a few of these tumors may be of muscle origin, but in the majority of cases the appearance is most probably ascribable to muscle damage. Lewis (*5*) showed that on transplantation of sarcomas with muscle elements, the modified muscle elements with rare exceptions disappear in later transplant generations. He suggested that either the muscle cells are lost or die out, or that they are malignant but dedifferentiate into spindle cells. In this series, metastases from one sarcoma with muscle-cell elements were found in the lungs and contained only the sarcoma cells (fig. 1, *A*, and *B*).

Carcinomas

Carcinomas were found in 1 percent of the tumors. In two, the total tumor was composed of carcinoma, and in two, the epithelial tumor was mixed intimately with spindle-cell sarcoma. There was no external ulceration preceding the develop-

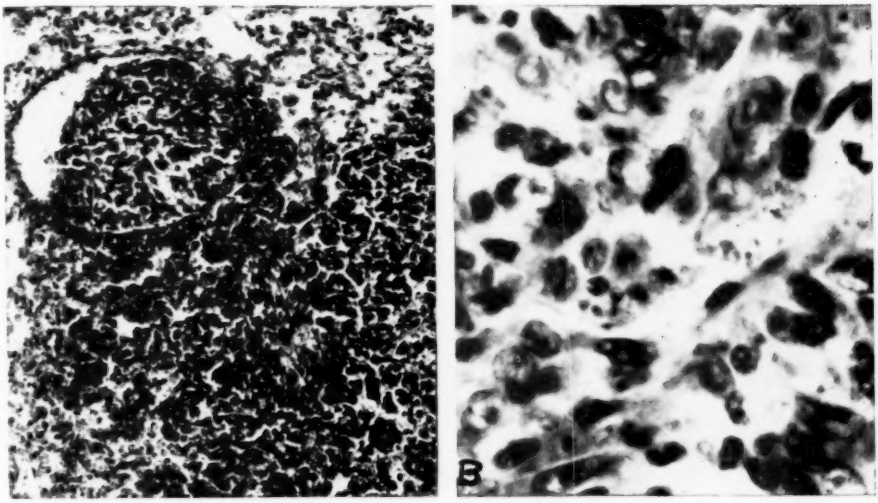

FIGURE 1.—*A*, Pulmonary metastasis of subcutaneous sarcoma with prominent muscle-cell elements, showing tumor embolus and growth in tissue. Haematoxylin and eosin, × 135; *B*, Higher magnification of *A*, showing absence of muscle-cell elements. × 465.

ment of these tumors, and grossly they were not distinguishable from sarcomas.

The first tumor was a squamous-cell carcinoma that appeared 64 days after the injection of 0.5 mg. of methylcholanthrene. No sarcoma was present, and the tumor consisted of solid masses of epithelial tumor cells. Keratinization was well-marked.

The second tumor was an adenocarcinoma, consisting of solid alveolar masses of polygonal cells and indistinguishable from a type of spontaneous mammary tumor frequently encountered in females of the C3H strain of mice. No sarcomatous elements were present. The tumor arose at the site of injection 75 days after the administration of 0.5 mg. of methylcholanthrene.

The third tumor was chiefly a fibrosarcoma, but in one location extending from a hair follicle was an area of squamous-cell carcinoma, with extensions penetrating into the fibrosarcoma. This tumor arose 83 days after the administration of 1.0 mg. of methylcholanthrene.

The fourth tumor arose 92 days after the injection of 2.0 mg. of benzpyrene and was composed of spindle-cell sarcoma, in the midst of which was a high grade carcinoma. The carcinoma was not in contact with any epithelial structures. The epithelial tumor was arranged in syncytial cords surrounded by a delicate reticulum. The cords alternated with endothelial-lined blood sinuses (fig. 2).

Neoplastic epithelial tissue was not encountered in the other 411 tumors, nor were squamous cysts seen; the cysts in the tumors were lined with either compressed sarcoma cells or with connective tissue fibroblasts. The skin over the tumor was often hyperplastic and keratotic, probably owing to reaction to the hydrocarbon. Mammary tissue of the male mice, which was included in some of the sections, was often hyperplastic, whether encompassed in the tumor or peripheral to it. The second tumor de-

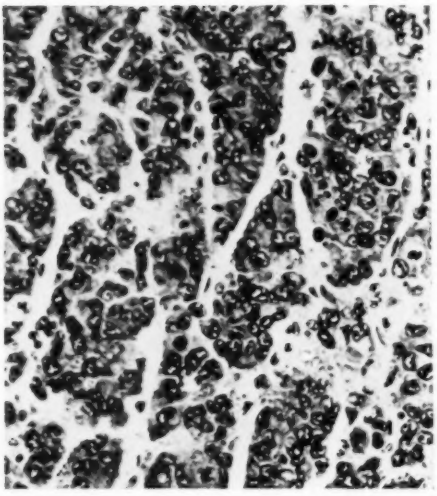

FIGURE 2.—Area of carcinoma in a spindle-cell sarcoma 92 days after injection of 2.0 mg. of benzpyrene. Haematoxylin and eosin. × 265.

scribed was the only one that developed from mammary tissue.

Other Masses

In four mice, the mass at the site of injection of the hydrocarbon consisted of hemorrhagic and necrotic tissue rather than neoplasm. These small masses showed almost no progressive increase in size and appeared in mice that received the lower doses of the hydrocarbons at a protracted period after injection (methylcholanthrene, 0.00098 mg. at 355 days, benzpyrene, 0.00195 mg. at 355 and at 375 days, and dibenzanthracene, 0.00195 mg. at 312 days).

The masses represented either late reaction to the solvent and the hydrocarbons or were early neoplasms that regressed. The second possibility is suggested by another small mass, obtained with 0.00195 mg. of benzpyrene, which was extremely hemorrhagic and necrotic but in which definite sarcoma was encountered at the periphery. Extensive hemorrhagic and necrotic areas were present in many other tumors, but they were uniformly large and often ulcerated through the skin.

RELATIONSHIPS BETWEEN MORPHOLOGY, LATENT TIME, GROWTH RATE, AND DOSE AND TYPE OF CARCINOGEN

The data on the latent time, growth rate, and the histologic appearance of the tumors were examined with respect to the following questions:

(1) Is the latent time and/or the growth rate influenced by the histologic appearance of the tumors within each dose and hydrocarbon group?

(2) Is the histologic appearance of the tumors influenced by the dose and the type of carcinogen employed?

(3) Is the appearance of multiple tumors influenced by the dose or the type of hydrocarbon employed?

(4) Is the growth rate of the tumors influenced by the dose and the type of hydrocarbon administered?

Latent Time, Growth Rate, and Morphology Within Groups

The analyses pertaining to the last three questions depend upon whether the tumors in a group of animals receiving the same dose of the same hydrocarbon can be considered as a whole, or whether it is necessary to subdivide each such group into histologic types before analyses are made of the relations between the other variables. Tumors of three general histologic types were induced: sarcomas, sarcomas with muscle-cell elements, and carcinomas with or without sarcoma. Since three sections were taken, at the most, through each tumor and since there is great overlap in the designation of sarcomas with reference to the amount of muscle-cell elements they contain, the classification cannot be considered as

highly accurate. Nevertheless, it is important to determine whether these general classes of tumors behave differently with respect to induction time and rate of growth.

The tumors of each dose group of each hydrocarbon were therefore divided into the three general morphologic classes, and comparisons were made between the latent times and growth rates of the different types of tumors. Statistical analyses showed no significant differences between the latent times or between the growth rates of tumors in the two morphologic groups, sarcomas, and sarcomas with muscle-cell elements. The tumors in the third morphologic group, carcinomas, were too few for satisfactory statistical comparison, but all of the growth rates as well as the latent periods fell within the limits observed for the other groups.

A second within-group analysis of importance concerns the possible association between latent periods and rates of growth. The variations in both growth rate (see section entitled "Growth Rate, Dose, and Type of Hydrocarbon," p. 32) and latent time (*1*) were relatively wide within individual close groups, and no significant correlation could be demonstrated between the length of the latent period and the rate of tumor growth. Brues et al. (*7*) also failed to find significant differences in growth rate between the early and late appearing tumors of a single series.

It is concluded that within dose groups the histologic variations did not exert significant influence upon the latent time and growth rate, and that the growth rate was not to a significant degree dependent upon the latent period. All tumors in a given dose group, therefore, were considered as belonging to the same population of variates despite differences in the histologic type and in the latent time and growth rate.

Morphology and Dose and Type of Carcinogen

Analysis of the types of tumor induced with the various doses of the three hydrocarbons (table 1) showed that the percent of sarcomas with prominent muscle elements is higher with the higher doses of the hydrocarbons. If the dose groups of the three hydrocarbons are arbitrarily divided at the mid-point of the effective doses, the incidences of sarcomas with prominent muscle elements are as given in table 2.

Table 2.—*Incidence of sarcomas with prominent muscle elements in C3H male mice injected with 3 hydrocarbons*

Hydrocarbon	Dose	Total tumors	Sarcomas with prominent muscle elements
	Milligrams	*Number*	*Percent*
Methylcholanthrene	1.0 −0.125	82	42.7
Do.	.0526− .0078	38	26.3
Benzpyrene	8.0 −1.0	69	47.8
Do.	.5 − .0039	52	21.2
Dibenzanthracene	8.0 − .5	92	42.4
Do.	.25 − .00195	82	17.1

It is probable that the larger doses of the hydrocarbons produce greater injury which results in greater alterations in the muscle. The resultant tumors are identical with sarcomas which do not contain muscle elements, as far as latent time and growth rates are concerned.

The four tumors consisting of, or containing carcinoma were induced with the larger doses of the more rapidly acting hydrocarbons. The latent periods were relatively short. They fall, however, within the range of variability of the latent period of sarcomas induced with the particular dose of the hydrocarbons.

In two previous series (*8*, *9*) in which 0.1 to 3.0 mg. of the three hydrocarbons were injected in tricaprylin, the incidence of carcinomas or mixed carcinoma-sarcomas was 4 of 359 tumors (1.1 percent).

The present incidence, 0.96 percent, is not significantly different. This incidence of epithelial tumors in strain C3H males probably is dependent upon the peculiar biologic constitution of this particular inbred stock of mice. In male and female mice of NH strain studied by Strong (*10*) the distribution of 348 tumors induced by subcutaneous injection of 1.0 mg. of methylcholanthrene in 0.1 cc. of sesame oil was as follows: sarcomas, 209; carcinomas, 83; and mixed tumors involving both carcinoma and sarcoma, 56. Thus, the incidence of tumors consisting of, or containing carcinoma in the NH strain was 40 percent, as contrasted with 1 percent in the C3H strain.

Multiple Tumors

Twenty-two of the four hundred and fifteen animals with tumors had more than one separate tumor. Twenty mice had two distinct masses. Two had three masses each; of these, one was injected with 0.25 mg. and the other with 0.06125 mg. of methylcholanthrene. The distribution of the multiple subcutaneous tumors is given in table 1. All of the tumors were sarcomas, and approximately half contained muscle elements.

The data are not sufficient to establish statistically whether there is a correlation between the occurence of multiple tumors and the dose or the type of hydrocarbon. From inspection, it is apparent that there is no significant correlation between dose and the incidence of multiple tumors in any of the hydrocarbon groups.

The incidences of mice with multiple tumors as the percent of the total number that developed tumors with each hydrocarbon are as follows:

Methylcholanthrene	8	of 122,	or 6.5 percent
Benzpyrene	8	of 127,	or 6.3 percent
Dibenzanthracene	6	of 184,	or 3.3 percent

The differences are not significant statistically, but it is of interest that the trend is in order of the speed of action of the hydrocarbon. Furthermore, methylcholanthrene, the most rapidly acting compound, was the only one to produce three tumors in a given animal.

It is probable that the appearance of multiple tumors is due in part to the area with which the injected carcinogen comes in contact. This depends to a great extent upon the amount of solvent used. In this series, 0.25 cc. of tricaprylin was used in all instances except for the highest doses of methylcholanthrene and dibenzanthracene, in which 0.5 cc. was used. The incidence of multiple tumors in the whole series was 5.1 percent. In a previous study (*9*), 63 mice were injected with methylcholanthrene or dibenzanthracene, 0.5 and 0.1 mg. dissolved in 0.1 cc. of tricaprylin. The incidence of multiple subcutaneous tumors was 3.2 percent (2 mice).

Growth Rate, Dose, and Type of Hydrocarbon

Preceding analyses showed that despite variations in histologic type and latent periods between individual tumors, the tumors within each group can be considered as belonging to the same population of variates with respect to growth. The data were, therefore, examined for correlation between the growth rate of the tumors and the type and dose of hydrocarbon.

The average growth rates, in terms of K, are given in table 1. Figures 3, 4, and 5 show the individual tumor growth rates plotted against dose. The range of doses was wide, and a logarithmic scale was used for convenience in plotting the figures.

In the instance of dibenzanthracene (fig. 5) the variation in growth rate between dose groups is not significant as compared with the variation within groups

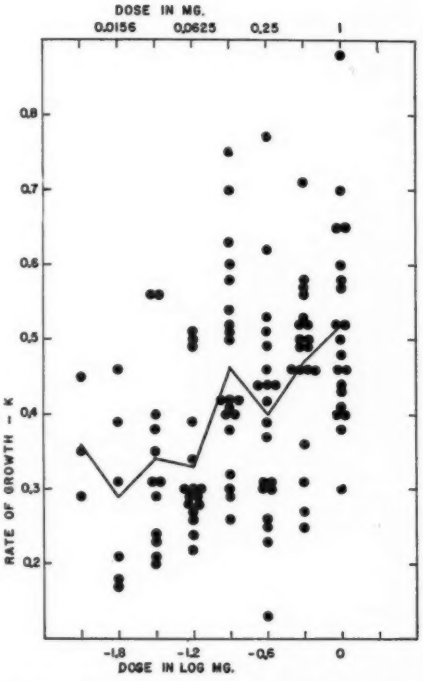

FIGURE 3.—Rate of growth of subcutaneous tumors induced with methylcholanthrene. Circles represent individual observations, plotted to avoid superposition; the line connects the average growth rates of the dose groups.

($P>0.05$).[4] The growth rates for methylcholanthrene and benzpyrene (figs. 3 and 4), however, vary significantly among the respective groups ($P<0.01$). The data are too variable to permit conclusions regarding the nature of the growth rate-dose regression curves, but the general trends of the data are shown in the figures by the lines connecting the group means. The trend of the methylcholanthrene data is clear cut and indicates that on the average tumors produced with the higher doses grew faster than did those produced with the lower doses. The trend of the benzpy-

[4] *The F statistic* (11) *was used in making the analysis of variance.*

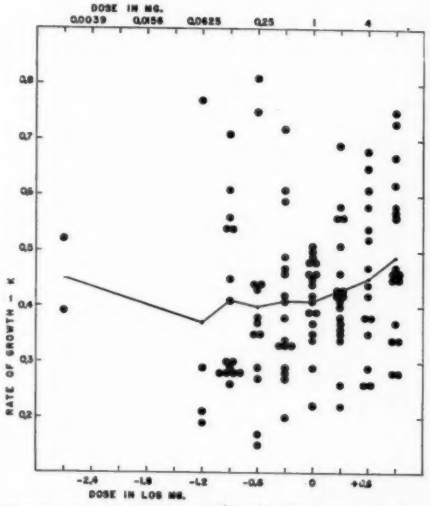

FIGURE 4.—Rate of growth of subcutaneous tumors induced with benzpyrene. Circles represent individual observations, plotted to avoid superposition; the line connects the average growth rates of the dose groups.

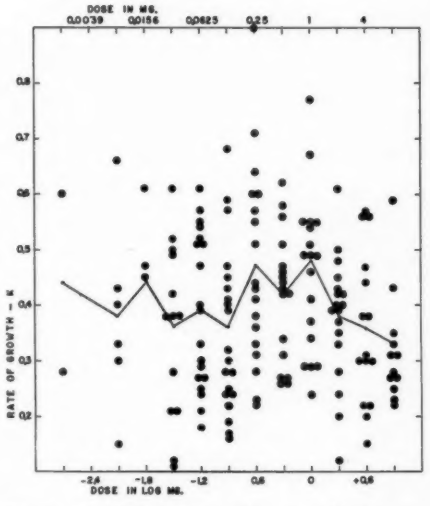

FIGURE 5.—Rate of growth of subcutaneous tumors induced with dibenzanthracene. Circles represent individual observations, plotted to avoid superposition; the line connects the average growth rates of the dose groups.

rene data is not so obvious. The maximum difference between group means is much less than that found with methylcholanthrene.

It is apparent from the data on the individual observations, however, that there is great variation in the growth rate, and that many tumors induced with smaller doses of methylcholanthrene and benzpyrene grew more rapidly than did others induced with larger doses.

Brues et al. (*7*), who first recorded that tumors induced with higher doses of carcinogenic hydrocarbons grew more rapidly than those induced with lower doses, correlated the growth rate with the speed of response. They postulated that the growth rate of a tumor in the microscopic stage may be the main factor in determining the latent period in carcinogenesis, and that variations in latent period might be owing to the number of malignant cells originally produced. Blum (*12*), studying the induction of tumors by ultraviolet radiation, pointed out that both rate of tumor cell proliferation and initial number of tumor cells must affect the time required for appearance of tumors. He found it necessary to postulate an additional factor regulating the proportionality between number of cells present at a given time and number of cells produced.

The induction of tumors, both from the standpoint of incidence and the length of the latent period, is probably primarily dependent upon the magnitude of the stimulus (dosage) and upon the length of time the stimulus acts. The factor of dosage is brought out in the present investigation. The importance of the length of action of the stimulus has been reported by Andervont and Shear (*13*), who showed that when methylcholanthrene-cholesterol pellets are implanted in C3H male mice the percentage of tumors elicited and the latent time are directly proportional to the length of time such pellets are permitted to remain in situ. The number of malignant cells originally produced is probably of importance in determining the incidence of tumors as well as the latent period. A small number of malignant cells may not become established and develop into a gross tumor, or the local or general resistance of the host may overcome the process. The latter is suggested by the appearance of necrotic, hemorrhagic masses at the site of injection at protracted periods after the administration of extremely low doses. The masses may well be regressed tumors.

In both this series and the investigations of Brues et al. (*7*), the growth rate of tumors was perforce measured after the gross appearance of the masses. Therefore, measurements were commenced when the tumors had reached approximately the same size. Despite this, the tumors induced with larger doses of the hydrocarbons grew more rapidly. Brues showed that the percentage of cells in mitoses was higher in these more rapidly growing tumors. This suggests that the carcinogen exerted a stimulating effect upon the tumor. It is possible, however, that the more rapid increase in tumor size with the high doses is in a larger measure owing to the addition of newly formed tumor cells as a result of the continual action of the carcinogen on surrounding tissues.

A previous study showed that the mean length of time between the detection of subcutaneous tumors by palpation and death of C3H mice, 4.36 ± 0.10 weeks, was the same whether methylcholanthrene or dibenzanthracene was used and whether the dose of the carcinogen was 0.1 or 0.5 mg. With a wider spread of dosage a trend toward longer survival may be anticipated with tumors induced with lower doses. However, the data are sufficient, if gaged against the present observations on growth, to suggest tentatively that the

death of an animal with a tumor is dependent not only upon the size of the tumor but also upon the length of time such tumor has been present in the animal. The death of the animal may be ascribable to the toxic effects of the neoplasm as well as its mechanical interference with motion and other functions.

SUMMARY

Of 415 subcutaneous tumors induced by 8.0 to 0.00195 mg. of methylcholanthrene, dibenzanthracene, and benzpyrene in male mice of the C3H strain, 411 were spindle-cell sarcomas; 2 were carcinomas, and 2 consisted of mixed carcinoma and sarcoma. One-third of the sarcomas had prominent muscle-cell elements.

Histologic variations did not exert a significant influence upon the latent time and growth rate of the tumors.

Prominent muscle-cell elements appeared more frequently in tumors elicited with the higher dose of the carcinogens. The four carcinomas appeared relatively early after the injection of the larger doses of methylcholanthrene and benzpyrene.

Subcutaneous sarcomas that were induced with larger doses of methylcholanthrene and benzpyrene grew more rapidly, on the average, than did tumors induced with smaller doses.

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Ultracentrifugal Studies of Some Complexes Obtained From Mouse Milk, Mammary Tumor, and Other Tissues

By HERBERT KAHLER, *senior biophysicist*, and W. RAY BRYAN, *biologist*, with the technical assistance of H. M. SIPE, *scientific aide*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Recent investigations showed that the mammary tumor inciter, present in milk of high-mammary-tumor-strain mice (*1*), could be sedimented by ultracentrifugation at 110,000 g for 60 minutes (*2*) but that an appreciable quantity of the active agent under different conditions remained unsedimented after 60 minutes at 60,000 g (*3*). It was also found[1] that the similar mammary tumor inciter present in saline extracts of mouse-mammary-tumor tissue (*3*) could be sedimented in the ultracentrifuge. Orienting experiments[2] made on both milk and saline extracts of mammary tumors were consistent with the possibility that the tumor-inciting activity was associated with constituents having sedimentation rates somewhere between 25 and 200 *s* (where $s=10^{-13}$ c. g. s. units). While these qualitative tests were not designed to answer the question whether the activity could be assigned to a definite sedimenting component within this range, nevertheless, they suggested that the activity with saline extraction was not owing to the large components in the 550 *s* class,[3] as is the case for the active agent from chicken tumor.

Claude (*5*, *6*, *7*) has shown that extracts of certain mammalian tumors, as well as those of normal chick-embryo tissues, contain macromolecular components which can be sedimented under the same conditions under which the active tumor-inducing agent of chicken tumor I is sedimented, i. e., centrifugation at approximately 20,000 g. We have observed similar macromolecular components in the materials of the present investigation.

In view of the relatively high centrifugal speeds required to give complete sedimentation of the mammary tumor inciter of mice, it appears probable that active fractions can be prepared which are relatively free of the larger macromolecular components such as those described by Claude. One of the chief purposes of the present investigation was to determine whether the various samples of milk and tumor extracts examined contained readily demonstrable macromolecular components having sedimentation rates between 25 and 200 *s*. Inasmuch as components falling within this range have been reported by Taylor, Sharp, Beard, and Beard (*8*) for extracts of normal chick-embryo tissue, additional observations were made on this material for the sake of comparison. These investigators showed that water extraction of the normal chick embryo gave a main component which had a sedimentation rate of about 80 *s*, for a total concentration of the centrifugal pellet material of 0.75 percent. Components in this range have also been found in extracts of normal silkworm

[1] *Unpublished experiments.*

[2] *Unpublished experiments.*

[3] *Stern and Duran-Reynals* (4) *obtained 532* s *for the normal chick component and 550* s *for the chicken-tumor component in 0.005 M buffer.*

tissues (*9*) and in the juice of certain pea and bean plants (*10*).

MATERIALS

The materials studied were: spontaneous mammary tumors of strain C3H mice (*11*); 11-day normal chick embryos; Jensen rat sarcoma (*12*) in Buffalo strain rats; transplants of mammary tumors in strain C mice (low spontaneous-mammary-tumor incidence); spontaneous mammary tumors in strain C mice[4]; milk of C3H (high-tumor-strain) mice; milk of C (low-tumor-strain) mice; unpasteurized cow's milk; and a single human mammary carcinoma.

In addition, optical centrifuge studies were made on pooled samples made up of equal parts of final ultracentrifugal fractions of normal-chick-embryo and C3H-tumor extracts.

EXPERIMENTAL PROCEDURE

The technique used for concentration and partial purification of the macromolecular components was essentially that described by Beard, Bryan, and Wyckoff (*14*) for isolation of the rabbit papilloma virus, except for the centrifugal speeds which were varied according to the component under consideration. One complete fractionation cycle consisted of a high-speed concentration and a low-speed clearance run in an ultracentrifuge. Table 1 shows the low and high speeds used in a given cycle for each of the two fractionation techniques employed. Technique I was used to study the larger particles such as those described by Claude. Technique II was used for the study of the smaller particles. The materials were carried through one to five cycles depending upon the purpose of the experiment. The concentrated pellets thrown down at each high speed run were routinely resuspended in phosphate buffer pH 7.0 to 7.38, 0.02 to 0.04 M. The final pellet of a given fractionation series was resuspended in not more than 1 to 3 cc. of buffer. The final concentration of macromolecular material was 10 to 25 times that in the original milk or tissue extract.

TABLE 1.—*Centrifugal speeds and fields employed in the fractionation of milk and tissue extracts*[1]

Technique	Concentration run			Multispeed clearance run		
	Centrifugal field	Speed (revolutions per minute)	Time	Centrifugal field	Speed (revolutions per minute)	Time
		Number	*Minutes*		*Number*	*Minutes*
I	20,000 g	18,000	150	6,500 g	10,000	30
II	90,000 g	36,000	60	20,000 g	18,000	30

[1] Air-driven ultracentrifuge of the type described by Beams (*15*) used for fields of over 20,000 g. Belt-driven multispeed attachment of the International Centrifuge No. 2 used for fields of 20,000 g or less.

The mouse milk was obtained by means of a mechanical milking device (*16*) or by squeezing excised lactating mammary glands. The initial volume of milk employed for individual experiments was 10 to 20 cc. The extracts of tumor and other tissues were prepared by grinding the tissue in a mortar with sand and diluting four to five times its volume with 0.9 percent sodium chloride solution.

The analytical ultracentrifuge was of the Svedberg-Bauer-Pickels construction (*17*). The temperature was regulated by a thermostat-controlled water jacket, the rotor spinning in a hydrogen atmosphere of 1-cm. pressure. The optical system was of the Thovert-Philpot-Svensson design (*18, 19*); the speed was measured photoelectrically (*20*).

The sedimentation constant, S_{20}, was obtained by making optical runs with sufficient sodium chloride added to make the resultant 0.2 N in sodium chloride in order to suppress the primary charge effect.

[4] *A high-mammary-tumor line of the resistant C strain was produced by foster nursing the progenitors in infancy on high-tumor-strain C3H females* (13).

TABLE 2.—*Summary of technical procedures and ultracentrifugal results*

Section	Date	Material	Extraction	Solvent in optical run		Technique	Cycle	Group			
								Slow		Fast	
				Buffer	NaCl molarity			S_{20}	Concentration	S_{20}	Concentration
		Mammary tumor in C3H mouse:						*S units*	*Percent*	*S units*	*Percent*
	June 30, 1942	Fresh	Saline	7.02	0.2	II	4	59.1	0.37	86.6	0.2
	July 1, 1942	do	do	7.02	.2	II	5	59.5	.12		
A [1]	Aug. 9, 1942	do	do	7.02	.2	II	2	54.4	.86	77.6	.62
	Aug. 15, 1942	do	do	7.02	.2	II	2	45.4 49.6	.59 .91	[2] 67.0	.75
	Apr. 24, 1942	Glycerolated	do	7.0	.2	II	3	55.1	.26		
B [1]	Aug. 26, 1942	do	do	7.02	.2	II	3	55.4	.27	84.4	.11
	Aug. 27, 1942	do [3]	do	7.02	.2	II	3	51.6	.47	86.0	.27
	July 7, 1942	Normal chick embryo	do	7.02	.2	II	2	62.1	.2		
	Aug. 3, 1942	do	Water		.2	II	2			90 113	
	Aug. 9, 1942	do	Saline	7.02	.2	II	2	52.2	1.49		
C [1]	Aug. 15, 1942	do	do	7.02	.2	II	2	51.6	.87		
	Aug. 26, 1942	do	do [4]	7.02	.2	II	3	50.6	.41		
	Sept. 1, 1942	do	do	7.02	.2	II	2	43.4	1.55		
	Nov. 25, 1942	do	Water		Water	II	2			89 141	
	Aug. 8, 1942	Pooled C3H tumor and chick embryo.	Saline	7.02	.2	II	2	62 69	.26 .58	90.0	.43
D	Aug. 15, 1942	do	do	7.02	.2	II	3	45.2 63.6	.43 .58	78.6	.25
	Aug. 26, 1942	do	do [4]	7.02	.2	II	3	49.7	.70	83.9	.20
	Sept. 1, 1942	do	do	7.02	.2	II	2	42.2 45.2	1.3 .6	(55.5)	.25
	May 21, 1942	Jensen rat sarcoma in Buffalo rats.	Saline	7.05	.2	II	[5] 3	43.4	.13	72.8	.13
E [1]	May 22, 1942	do	do	7.05	.2	II	[6] 3	46.3	.12		
	May 29, 1942	do	do	7.05	.2	II	3	41.5	.19	87 115	
	May 30, 1942	do	do	7.05	.2	II	3	42.7	.20		
F	June 15, 1942	Transplanted tumor in C mouse.	do	7.02	.2	II	2	49.7	1.34	57.8	.87
	June 16, 1942	do	do	7.02	.2	II	3	51.7	1.25	69.8	.75
	June 17, 1942	do	do	7.02	.1	II	[5] 3	54.3	.27	92.2	.12
G	May 8, 1942	Spontaneous tumor in C mouse.	do	7.05	.2	II	2	59.7	.68	93.3	.20
		Milk:									
	Oct. 16, 1941	Squeezed; C3H mouse		7.38	.2	I	1	42.2 55.3	.07 .38		
H	Oct. 17, 1941	Squeezed; C57 mouse		7.38	.2	I	1	48.4 58.6	.25 .27		
	Nov. 7, 1941	Machine; C3H mouse		7.38	.2	I	1	63.5	.34		
	June 19, 1942	Squeezed; C mouse		7.02	.2	II	2	(30) 50	.09	(75)	
J	June 19, 1942	Cow, unpasteurized		7.02	.2	II	2			73.2	.45
K	Apr. 21, 1942	Human mammary carcinoma.	Saline	7.0	.2	II	[7] 1	40 54		75.4	
L	Sept. 27, 1941	Mammary tumor in C3H mouse.	do	6.8	.2	I	1			79	
	Sept. 29, 1941	do	do	6.8	.2	I	2			184	
	Oct. 11, 1941	do	do	7.35	.2	I	1			41	.79
M	Oct. 13, 1941	do	do	7.35	.2	I	1			76	.42
	Oct. 14, 1941	do	do	7.35	.2	I	2			183	.43
	Sept. 19, 1941	do [4]	do	6.8	.2	I	6			214	
N	Sept. 22, 1941	do	do	6.8	.2	I	6			145	
	Sept. 24, 1941	do	do	6.8	.2	I	6			72	
O	Dec. 2, 1942	do	Water	7.02	.2	{Special.}	2			93 193	

[1] Shown in graph, fig. 1.
[2] See text, footnote 6.
[3] ½ dilution.
[4] Glycerolated.
[5] 3 cycles + 1 additional clearance run.
[6] 3 cycles + 2 additional clearance runs.
[7] 1 cycle + 1 additional clearance run.

The usual method of correcting for temperature, viscosity of solvent, and partial specific volume of the component was employed. The value for the partial specific volume, as used by Svedberg and Pedersen (*21*) for proteins, was uniformly used. The recently published value of Taylor, Sharp, Beard, and Beard (*8*) for the nor-

mal-chick-embryo-component density, 1.27, if used would raise the sedimentation constants by 3 percent. Claude (*22*) gave a density value of 1.3 for the largest components, while Stern and Duran-Reynals (*4*) gave a density value of 1.23 for the chicken-tumor component.

The concentrations of the various components were determined by planimeter measurement of the areas under the sedimentation diagrams. For calibration purposes a known weight of material was employed, use being made of Svensson's correction formula (*19*).

RESULTS

The results are given in table 2, in which are included concentration and sedimentation constants for the materials studied.

The fact has been well-established by numerous investigators that certain molecules sediment at a faster rate in dilute solutions than in more concentrated solutions. The practice has frequently been followed of plotting inverse sedimentation against concentration and extrapolating the curve so obtained to infinite dilution. The reciprocal of the value obtained at infinite dilution when corrected to standard conditions is the sedimentation constant. No adequate theory of the concentration effect has been given in terms of molecular shape. An alternative empirical procedure, used in this study, was to plot concentration against sedimentation rate directly, and from this plotting to obtain the sedimentation constant at vanishingly small concentrations.

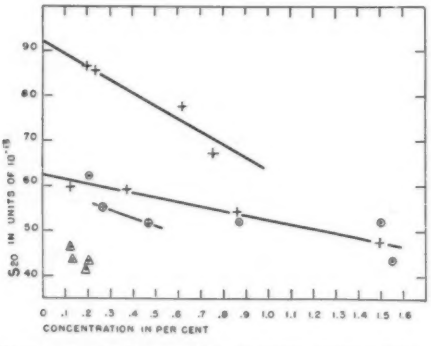

FIGURE 1.—Sedimentation constants against concentration of component in percent, for various materials.

The concentration of component was plotted against S_{20} for some of the materials studied (fig. 1). The C3H tumor extract gave a series of points defining a line which cuts the zero axis at 62 *s* for the slowly sedimenting component and at 90 *s* [6] for the rapidly sedimenting component. If the higher value (*8*) for partial specific volume were used, these points would be about 64 and 93 *s*, respectively. For the slow component the sedimentation values for the normal chick embryo fall reasonably close to those for the C3H tumor material.

The fast component for the normal chick embryo (fig. 2) is of negligible concentration, whereas for the C3H tumor material under similar treatment the fast component has an appreciable concentration (fig. 3).

The C3H tumors that had stood in glycerin for from 5 weeks to 4 months gave a lower sedimentation rate for the slow component. From parallel biologic studies [7] it was established that tumor material glycerolated for 4 months had lost all of its activity.

The values for the pooled material did not fall on the C3H-chick line in all cases (fig. 4 and table 2). For some concentrations the pooled material moved faster

[6] *Low values such as the one for 0.75-percent concentration for the fast component are to be given less weight, since the fast component moves in a solvent of which the slowly moving material is a constituent. The resulting increase in viscosity, for which no correction was made, is not negligible in this instance since the slow component had a concentration of 1.5 percent. A corrected value at 0.75 percent would cause the line to cut the zero axis at nearer 90 than 92* s.

[7] *Unpublished data.*

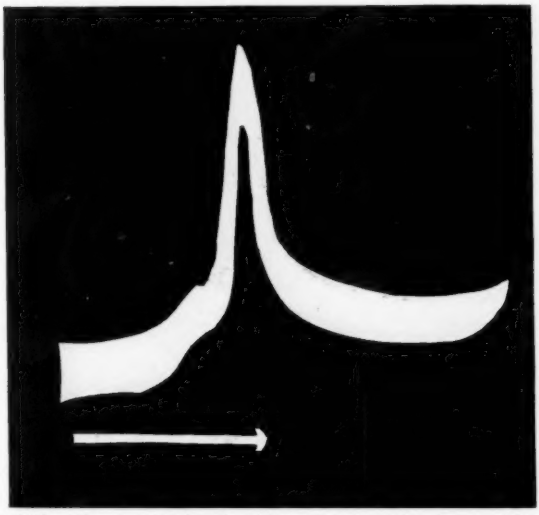

FIGURE 2.—Normal chick embryo, second-cycle saline extraction, showing the 62–64 *s* band. There is little evidence of a faster moving component (Tracing).

than would be expected if the C3H and chick components were identical. Presumably there was some intercombination of the components that resulted in a faster moving particle. (In this connection, see Svedberg and Pedersen's data (*21, p. 409*) on combination between lactoglobulin and serum albumin showing intercombination at different concentrations.)

A much lower series of values was obtained for the main slow component of the Jensen rat sarcoma (fig. 1).

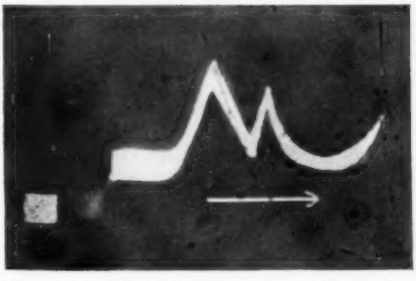

FIGURE 3.—Fresh C3H tumor-material extract, second-cycle saline extraction. The slow component is the 62–64 *s* band, the fast component the 90–92 *s* band (Tracing).

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The data given for sections L, M, and N (table 2), which were obtained with technique I, show that both aggregation and splitting occurred during the handling of the material. Water extraction and handling of material (C3H tumor), using a 12-minute clearance run of only 10,000 g gave two sharp bands (fig. 5) with most of the material in the slower component. The following day another aliquot was studied using 0.2 N sodium chloride during the run. Figure 6 indicates that the faster moving component now comprised the major part of the material. This is evidence of aggregation.

Since water extraction gave a high percentage of the material in faster sedimenting components which were evidently part of a series of complexes, the question arose whether activity would be carried by these larger aggregates. A preliminary test[8] using water-extracted C3H tumor material indicated that the greater part of the tumor-inducing activity was associated

[8] *Unpublished data.*

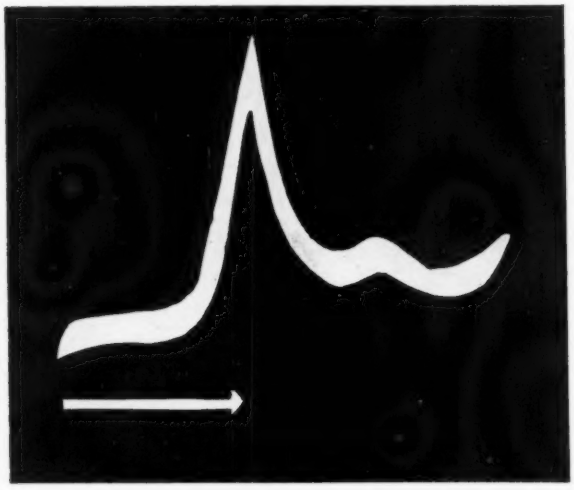

FIGURE 4.—Sample made by pooling equal parts of C3H tumor (fig. 3) and normal chick embryo (fig. 2.).

with the larger components. This suggests that activity may be associated with several of the elements of this series of complexes.

In this connection, it may be pointed out that the more rapid sedimentation indicated by the data of Visscher, Green, and Bittner (*2*) compared with that of this laboratory (*3*) may be owing to the use by the former authors of milk diluted one to two times with distilled water thus giving particles of higher aggregation.

At high concentrations (greater than 1 percent) the C3H tumor material gave a slowly sedimenting group that could be resolved into two peaks differing in sedimentation rate by 7 percent (fig. 7). The

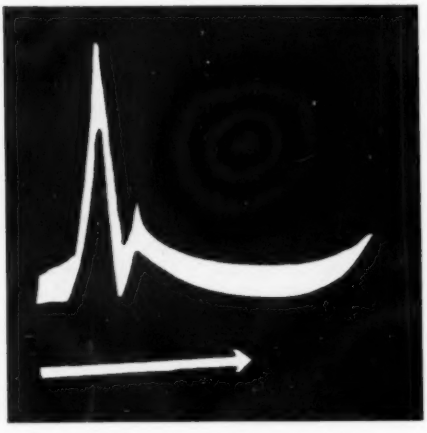

FIGURE 5.—Water-extracted fresh C3H tumor material. Photograph was taken of the pellet suspension to which no salt was added (Tracing).

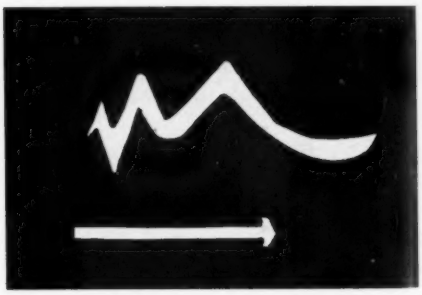

FIGURE 6.—Water-extracted fresh C3H tumor material, same as in figure 5, after standing in water 1 day. Slow band S_{20}, 93; large fast band S_{20}, 193. The slower band and peak here correspond to fast peak in figure 5 (Tracing).

chick embryo at similar concentrations did not show the same effect, the band remaining single throughout the course of the sedimentation run. In figure 1, the mean value for these two C3H components was plotted against the total concentration, at the high-concentration end of the graph.

Milk was examined with techniques I and II, but no large amount of homogeneous material with larger *s* rates than the tabulated components was present. The three samples of milk (obtained by squeezing), which contained substances derived directly from the tissue, gave *s* values for the slower component similar to those for the mammary tumor extracts but also an additional slower moving component. The C3H mouse milk obtained with the machine showed a single slow component with some aggregation among larger components. Mouse milk seems to be relatively deficient in the fast component.

Ultraviolet spectrophotometric studies were made on the ultracentrifuged fractions of some of these materials (fig. 8). The fractions from C3H mammary tumor and pressed C mouse milk showed a strong absorption maximum at 2600A, which is indicative of the presence of nucleic acid. The C3H milk obtained with the milking machine, however, showed a relatively broad plateau from 2600A to 2800A, which indicated a higher ratio of protein to nucleic acid content. Cow's milk gave a sharp maximum at 2800A, which indicated protein only.

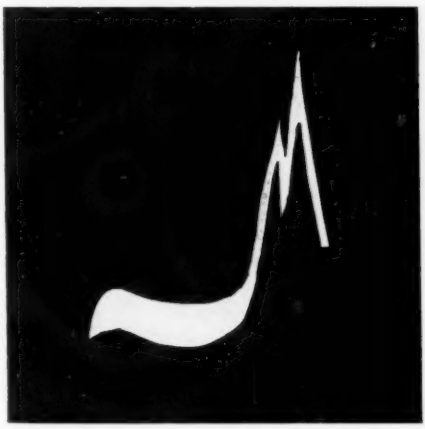

FIGURE 7.—High concentration of C3H tumor material showing resolution of slow band into two components (Tracing).

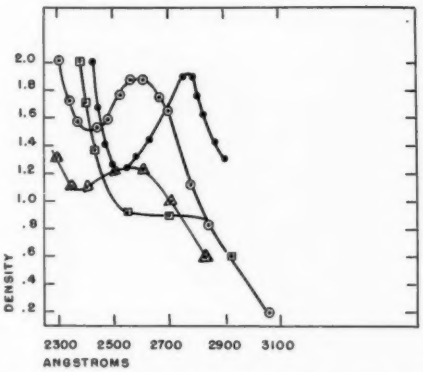

FIGURE 8.—Ultraviolet spectral absorption. Solid circles: Cow's milk, second-cycle pellets; Open circles: Milk squeezed from low-tumor-line strain C mice. This milk contained fluid pressed from tissues, second cycle; Squares: Machine milk from strain C3H mice, second cycle; Triangles: C3H tumor extract, fifth cycle.

Bial's test, made by Dr. Robertson, of the National Cancer Institute, on C3H tumor pellet material showed a positive reaction for pentose, indicating that this material may be a ribose-nucleic complex. These results are similar to those described for the normal chick embryo(*8*), which was found to be a ribose nucleoprotein combined with lipoids.

A sedimentation constant of 62 to 64 *s* for the principal saline-extracted constituents would indicate a minimum molecular weight between three and four million (on a spherical-shape basis). For the fast component, 90 to 92 *s*, a minimum molecular weight of five million would be expected.

The variation of sedimentation constant with concentration of component as shown in figure 1 is evidence which indicates that the macromolecular particles are of a nonspherical shape. It has been found (*23*), for instance, that nucleohistone with a molar dissymmetry constant of $\frac{f}{f_0}=2.5$ varies in sedimentation rate from 14 at 0.5-percent concentration to 32 at nearly zero concentration. On the other hand, spherical particles $\frac{f}{f_0}=1$ show little or no change in sedimentation rate with concentration. If the particles are not spherical in shape, the molecular weight would be larger than the minimum values given herein.

SUMMARY

By utilizing the differential centrifugation method, it was found that in certain tissues and mouse milk the chief constituents showing sedimentation values between 25 *s* and 200 *s* are the nucleoprotein (lipoid) complexes.

The sedimentation rates of two principal components for C3H tumor extract when extrapolated to zero concentration are 62 *s* and 90–92 *s*. If an alternative value for partial specific volume were used, these values would be increased by 3 percent.

The normal chick-embryo values for the slow component fall very close to those for C3H tumor. Under the saline-extraction conditions used, however, the faster moving component in the chick embryo was of negligible concentration.

The fast component in C3H mammary tumor is evidently the analogue of the main component obtained from normal chick embryo by water extraction.

Somewhat similar components were found in transplanted mammary tumors in strain C mice and the milk of three strains of mice resistant and nonresistant to spontaneous mammary tumor. Human mammary carcinoma gave indications of somewhat similar components in this range.

Cow's milk and Jensen rat sarcoma gave different values from those of mouse mammary tumor and milk.

A minimum molecular weight of three to four million was estimated for the macromolecular materials of 62 *s*, which was the principal component of chick embryo and mouse mammary tumor, while the faster moving component had a molecular weight of at least five million. The variation of sedimentation rate with concentration indicates an asymmetric particle.

Studies on concentrated suspensions of C3H mammary tumor material showed that the fraction giving the main sedimentation peak could be resolved into two neighboring components differing by 7 percent in sedimentation rate. High concentrations of normal chick-embryo material did not show a resolved double peak. The main component consisted of a sharp band.

Pooled C3H-tumor and chick-embryo materials did not give *s* values falling on the C3H-chick plot for all concentrations. This suggests intercombination between components not chemically identical.

Glycerolated C3H tumor material several weeks old gave smaller sedimentation values than did fresh material.

Evidence for the existence of both aggregation and dissociation reactions is given. This is an extension of the findings of others on chick embryo.

Ultraviolet absorption spectograms showed a high percentage of nucleic acid in the fraction from mammary tumor and pressed milk, considerably less in machine-obtained milk and Jensen rat sarcoma, and none in cow's milk. Bial's test showed a positive reaction for pentose. This

finding, combined with the ultraviolet absorption, indicated the presence of a ribose-nucleic acid complex in the material from C3H mammary tumor.

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Method for the Quantitative Morphologic Analysis of Tissues

By H. W. CHALKLEY, *senior physiologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

A perennial technical problem in histology and pathology is the difficulty of obtaining quantitative data on the spatial distribution of morphologic tissue components and their changes in growth, functional activity, and disease. It is hoped that the method here presented will prove of use in this respect.

The requirements for such a method are (1) that it be sufficiently accurate, (2) that it be applicable to tissues of considerable complexity, (3) that it be simple, and (4) that it be possible to subject it to independent check by other methods when necessary. The method proposed fulfills these requirements within reasonable limits. Heretofore, the method mainly depended on has been the transfer of the outlines of the microscopic image of the tissue component of a section, as seen on the slide, to paper either by means of a camera lucida or by photography, with subsequent measurement of the components of interest, such as cells, nuclei, etc., by use of the planimeter or by the excision and weighing of the outlined areas. This method is at present commonly limited to the measurement of cells and nuclei and is not used for even semi-routine procedure by reason of the inordinate amount of time required. What is ordinarily required from such measurement is to secure, not the absolute area or volume of the components measured, but the ratio between the volumes or areas, particularly the nucleocytoplasmic ratios, or the volume ratios of active and inactive gland cells. The proposed method directly ascertains such ratios and obviates the necessity for tedious drawing and measurement.

METHOD

Let us consider a slice of tissue of relatively homogeneous structure such as a section of liver; let us also conceive of a mathematical point moving through that tissue and following a random path in three dimensions. It is obvious that the proportion of its path resting in the nuclei, in the cytoplasm, in the vascular spaces, or bile ducts will, if the path is indefinitely prolonged, approach as a limit the proportions of the fractions of volume occupied by these morphologic constituents.

To make the measurements of the path as described is, of course, impossible, but the problem can be approached quite simply in another way. By substituting for the continuously moving point a random distribution of points throughout the tissue and summing up the number of points lying in the various components, the ratios of these sums will also approach the ratios of the volume fractions occupied by the respective components. The shape of the individual component units does not enter into this consideration.

If a method of ascertaining the sums of points as envisaged can be devised, the ratios between such sums can be ascertained for the components of a given unit area or volume; and if additional measures are obtained in respect to the components to give the number of units of each component in a given area or volume, the area or volume of each component unit can be

readily obtained. Hence, by using these additional measures it should be possible to check the results obtained with such a method against those acquired with the usual methods, which start by ascertaining the volumes or areas of the component unit and calculating the ratios. A description of the means of applying this concept and testing it against the usual methods follows.

The fundamental requirement is to obtain a random distribution of points within the tissue under observation. This can be done either by random scattering of points through the tissue, or random presentation of the tissue at a fixed point or a simple pattern of points. The latter procedure is the more practical for the purpose.

All that is required is to provide a means for indicating a point or points in the focal plane of a microscope image and for obtaining random movement in all dimensions of the specimen to be observed. The ordinary mechanical stage plus the focusing range of the microscope is sufficient for the latter, and the point or pattern may be obtained by cementing a hair or several hairs to the diaphragm of the ocular to act as finders. The hairs then appear in the focal plane of the image, and their free extremities constitute the point pattern. In practice the object is brought into focus, the position of the points noted, i. e., whether they fall on nucleus, cytoplasm, tissue space, etc., and these observations are recorded. The object is then thrown completely out of focus and by means of the mechanical stage is moved under the objective. It is then brought back into focus at varying vertical levels, and the position of the points is again noted and recorded. This process is repeated, following a fairly systematic pattern in all three dimensions and covering a sufficient portion of the tissue measured to give an adequate sample. It is not necessary to avoid the accidental repetition of measurement of the same space. Point-for-point repetition (unless deliberate) is almost impossible.

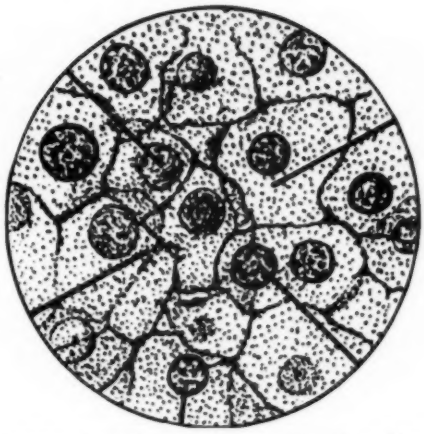

FIGURE 1.—Point pattern as seen in making measurements. The upper point is focused on a nucleus to serve as reference. The upper left point coincides in focus on a nucleus; the lower right one is over a nucleus, but the nucleus is not in focus. The other points are on cytoplasm. The reading is one hit on nucleus, three on cytoplasm.

In actual practice, a purely psychologic but very real difficulty arose. A trained observer, when using a finder such as the glued hairs, is accustomed to focusing in such a way that the finder coincides with some object. It was very difficult not to do this and thus bias the observations, especially when dealing with thick sections. To avoid this difficulty, a very short extra hair is glued on the eyepiece diaphragm; then the focus is so made that the tip of this hair is brought into coincidence of focus with a selected object, usually a nucleus. The position of this point is not recorded, but the other points alone are used. It is best to keep this short point at the upper limit of the field, since by so doing, the attention is concentrated there-

on and the positions of the other points are not noticed. As there is in this type of observation no significant relation between the position of this point and the others used, the positions of the points used are truly attained at random. The arrangement is shown in figure 1. As will be shown, this allows one to deal easily with thick or thin sections without introducing errors. (A method for the empirical determination of the number of observations necessary to constitute an adequate sample will be described later.) The "hits"[1] on each element are summed and the ratios calculated. The tests described in the following section show that satisfactory results can be obtained with this simple procedure.

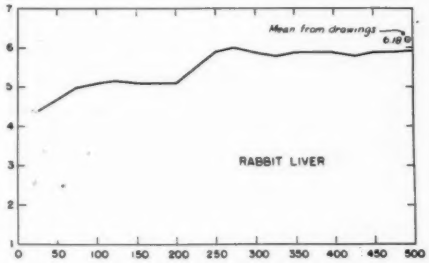

FIGURE 2.—March of the cumulative mean of the ratio of hits on nucleus to hits on cytoplasm of parenchymal cells on a section of rabbit liver, with increasing numbers of observations. Ordinate, ratio; abscissa, number of hits on nuclei. Note that the mean is stable with a total of 350 or more hits on nuclei. The ratio of average area of nucleus to cytoplasm as calculated from measurements of 50 camera lucida drawings agrees well with the ratio found.

TESTS

The first test of the method was made on a section of rabbit liver. The tissue preparation had been stained in such a manner as to show the cell outlines clearly; and this characteristic, since it enabled one to make camera lucida drawings, was used in checking the results. Observations were made by using a 20+ ocular and a 2-mm. oil-immersion objective, and the hits recorded on liver nuclei, liver cytoplasm, and all other components, these last being designated collectively "tissue space." The ratio of hits on cytoplasm to hits on nuclei was 5.82 (σ=1.22).[2] To check this result, camera lucida drawings were made from the same preparation of the outlines of 50 individual cells and their nuclei, selected for the ease with which the drawings could be made. The areas of all were measured with a planimeter, and the ratio between the average area of the nucleus and the cytoplasm was calculated. The value obtained was 6.2, σ1.52, a difference of 6.9 percent from the lower value. This seemed a satisfactory agreement considering the small number of cells measured.

In securing the data by the proposed method a sufficient number of observations was made to secure 500 hits on nuclei. To ascertain whether this number constituted a sufficient sample, the data were plotted to show the ratio as calculated from the successive totals of hits on nuclei and cytoplasm, as successive groups of 25 hits on nuclear material were obtained. A similar cumulative plot was made of the ratio for each successive group of cells and nuclei drawn and measured (fig. 2). The ratios thus plotted show less and less variation as the number of measurements accumulate, and it is fairly easy to judge to what extent they are likely to vary and thus to obtain an empirical estimate of the size of sample necessary for a reliable measurement.

If all measurements are so plotted and it

[1] *A "hit" is defined as the contact of the image of the point with the clearly observed image of the specimen.*

[2] *In all instances where the standard deviation is given for observations by this method, the deviations are those of a unit observation consisting of an arbitrarily determined group of hits, usually the number of hits per 25 hits on the element used as standard, in this case the nucleus.*

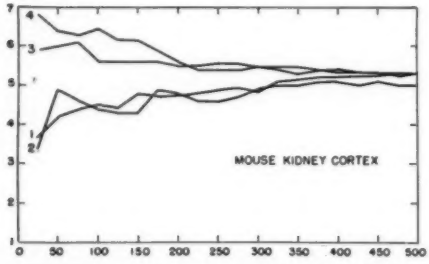

FIGURE 3.—March of the mean ratio of hits on nuclei to hits on cytoplasm of the tubule cells of a section of mouse kidney cortex for 4 sets of observations, each including 500 hits on the nuclei. Note the centering and stabilizing of the ratios with increasing number of observations, giving reproducible results.

is thus ascertained before applying statistical tests that a stable mean has been obtained, the effort of applying elaborate tests to intrinsically insufficient data is avoided.

In the next test of the method, that for reproducibility, a section of mouse kidney cortex was used. Bouin's fixation, paraffin embedding, and haematoyxlin-eosin stain were employed. Measurements were made of the tubule cells, and hits on nucleus, cytoplasm, and tissue space recorded. As before, all material other than tubal was designated tissue space. Four runs were made, and cumulative plots of the ratios made for each run (fig. 3). This shows that the method has a high degree of reproducibility on given material, the final nucleocytoplasmic ratios being 5.0, $\sigma=1.30$; 5.3, $\sigma=1.02$; 5.3, $\sigma=0.95$; 5.3, $\sigma=0.8$ for the respective runs, or an average spread of 4.4 percent of the lowest value obtained.

A further empirical test was made, that of the ability to deal with a section of a complex organ. A section of the mouse kidney was again used, and a record was kept of hits made on tubule cell nuclei, tubule cell cytoplasm, tubule lumina, glomeruli, and vascular space. The examination was confined to the cortex. The number of hits on the glomeruli was used as the index and the recording was carried on until 700 hits had been scored on glomerular tissue. The ratios were computed as between tubule cytoplasm and nucleus; tubule cells and tubule lumina plus glomeruli plus vessels; tubule cells and lumina; and tubule cells and glomerular tissue. The next step was to calculate the average areas of certain tissue elements, tubule cells, tubule lumen, and glomerulus. For the calculation it was necessary to have the following further measurements all at the magnification used: (1) The area of the tubule nucleus; (2) the area of the microscope field; (3) the average number of lumina per field; and (4) the average part of a glomerulus per field. The first two requirements were satified by making (1) camera lucida drawings of 600 nuclei, and (2) a similar drawing of the outline of the field, and measuring them with a planimeter. The last two requirements were met by counting the lumina and the glomeruli seen in a number of fields and dividing the total number of each by the number of fields counted. The results are given in table 1. It is obvious that all counts and drawings must be at the same magnification; or allowance should be

TABLE 1.—*Values obtained in analysis of mouse kidney cortex*

Description	Value	Standard deviation (σ)	Symbol
Area of microscope field	3,419		*A.*
Area of tubule nucleus	15.4± 0.12	2.92	*N.*
Ratio, Hits on tubule cells/Hits on other elements	5.51± .18	.94	*C.*
Ratio, Hits on tubule cytoplasm/Hits on tubule nuclei	5.38± .15	.83	*n.*
Ratio, Hits on tubule cells/Hits on tubule lumina	14.20± .87	4.60	*L.*
Ratio, Hits on tubule cells/Hits of glomeruli	15.70± .54	2.89	*G.*
Lumina per field number	1.31± .034	.136	*l.*
Gomerulus per field percent	5.38± .15	.83	*g.*

made for difference, if changes in this respect are made for reasons of expediency.

From the values given in table 1 it is possible to calculate (1) the average area of the tubule cell, (2) the average area of the tubule lumen, and (3) the average area of a glomerulus. They are as follows:

(1) $N(n+1)=$
the average area of the tubule cell,

(2) $A-\left(\frac{A}{1+C}\right)=$
the average area in tubules per field,

then $\frac{A-\left(\frac{A}{1+C}\right)}{L}=$
average area of luminal space,

and $\frac{\frac{A-\left(\frac{A}{1+C}\right)}{L}}{l}=$
average area of lumen.

(3) $\frac{A-\left(\frac{A}{1+C}\right)}{G}=$
average glomerular area per field,

then $\frac{\frac{A-\left(\frac{A}{1+C}\right)}{G}}{g}\times 100=$
average area of glomerulus.

These computations give predictions of the average areas of tubule cell, tubule lumen, and glomerulus. These values were checked independently by making camera lucida drawings of the outline of cells, lumina, and glomeruli, measuring them with a planimeter, and calculating their average areas. The results are given in table 2. The differences are all less than 5 percent of the lower values. This is a satisfactory agreement.

TABLE 2.—*Comparison of calculated and directly measured values for different elements of tissue section*

Element	Average area		Difference
	Calculated value	Measured value	
Tubule cell	98.2	[1] 99.5± 2.5	1.3
Tubule lumen	155.5	160.8±17.6	5.3
Glomerulus	1,644.6	1,602.8±44	41.8

[1] Large errors are due to the fact that comparatively small numbers were used in check measurements. The means were accepted as soon as they appeared reasonably stable in cumulative plot.

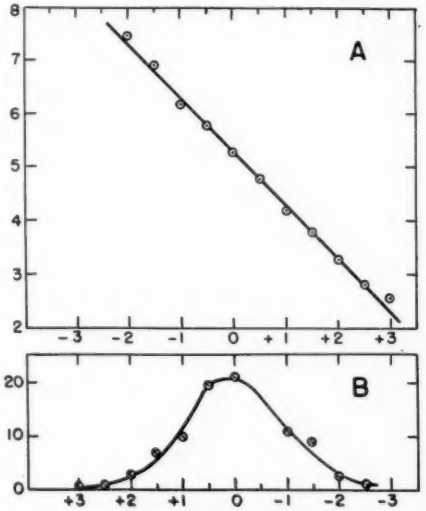

FIGURE 4.—Two plots of the distribution of the deviations about their means, of all unit observations in terms of deviation units. *A*, Probit curve; *B*, usual frequency distribution curve. Ordinate: *A*, probits; and *B*, number of deviations. Abscissa: both *A* and *B*, departure from mean values, positive and negative, in standard deviation units.

Two more tests remained to be made. The theory presumes a normal distribution of the variates. All variates were converted into standard deviation units and plotted. The distribution curve is shown in figure 4, *B*. An analyses was also made by the method of Bliss [3] to see if the distribution follows a normal curve. The integrated percentage frequencies were transformed to probability units (probits) and plotted against deviation in standard deviation

[3] *Bliss, C. I.: The comparison of dosage-mortality data. Ann. Appl. Biol., 22: 307–333 (1935).*

TABLE 3.—*Variation in nucleocytoplasmic ratio as calculated from measurements made on sections of differing thickness cut from the same block of mouse liver*

Thickness of section (in microns)	Ratio Cytoplasm / Nucleus	Difference
1		
3	10.10	0.04
5	10.56	.42
10	10.01	.13
15	9.90	.24
Mean	10.14	[1] .21

[1] Less than 3 percent of the mean.

units. The excellent fit to the straight line obtained by this transformation is shown in figure 4, *A*, and indicates that the frequency distribution of the variates does follow a normal curve. Since the hits are distributed in three dimensions, the measurements and therefore the ratio between two tissue components should be practically independent of the thickness of section used. This second test was made by measuring the hits on cytoplasm and nucleus in a series of sections of differing thickness cut from a single block of mouse liver. The results are given in table 3. The standard deviation is less than 3 percent of the mean, and the differences between the ratios found are evidently not related to the thickness of the sections.

ANALYSIS OF ORGANS

The method can readily be applied to the analysis of the whole or part of an organ. It has been applied with good results to the mouse anterior pituitary to ascertain the composition in terms of the morphologic components. It is thought that a sample of such an analysis may be of value, therefore the data for a single gland are presented herewith.

The material, furnished by Dr. Dalton, of this Institute, consisted of the complete serial sections of the anterior pituitary of a young adult male mouse of the C3H strain. Measurements were made on every ninth section, 8 sections in all; 2 sets of measurements were made on each section, each set being based on 25 hits on α nuclei per section. This was done to secure an adequate sample from the whole organ, since tests showed that an estimate based on a single section was totally inadequate. Hits were recorded for α cell cytoplasm, α cell nucleus, β cell cytoplasm, and β cell nucleus.

The cumulative plots of the means as percent of total hits are shown in figure 5. The greatest difference between any 2 sets of measurements on 1 component is less than 1 percent. This difference is exceptionally low, but from the data at hand (some 25 or more such measurements) the

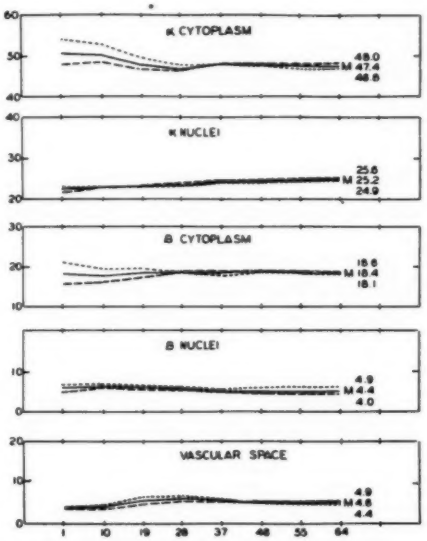

FIGURE 5.—Plots of the collective means of percentage of α cytoplasm, α nucleus, β cytoplasm, β nucleus, and vascular space for analysis of anterior lobe of a mouse pituitary. Dotted lines, march of means of groups of hits, giving 25 hits on α nucleus per section. Solid line, march of means of the 2 groups per section. Ordinates, percent of total hits scored. Ordinate scale, section number from serial sections cut at 3μ.

average standard deviation between such pairs of measurements is 1.8 percent. One can expect a standard deviation of definitely less than 4 percent. The time required to get such a series of measurements on 1 gland is less than 2 days. The data allow the calculation of several ratios, as between nucleus and cytoplasm, or cell and cell type, which would require a much longer time to obtain by any other method known to the writer.

DISCUSSION

It is necessary to emphasize that while the method permits calculation of such ratios as α cell to β cell, it does not give information about the ratio between the number of α cells and the number of β cells. The ratio obtained is that of the fractions of unit volume occupied by β cells to the fraction of unit volume occupied by α cells as determined by hits on β and α cells, respectively.

Most of the data of this sort in the literature deal with ratios between numbers of cells. In making comparisons between organs, the assumption is that all cells of a given type are alike in the organs compared. This the writer believes is probably untrue. The assumption itself is certainly fallacious.

In the method presented herein, no assumption as to number of cells or other components enters into consideration, the comparison being made between two or several fractions of volume, or of fractions of volume to total volume. This procedure appears to be preferable since it would seem a priori more likely that the physiologic, physical, or chemical significance of a component is directly linked to its proportional volume in a given organ or organism than to the number of compartments into which that volume is divided. If the latter information is needed, it can be readily obtained.

As in all experiments where a method of random sampling is used, care must be taken to design all experiments so that adequate samples are obtained.

SUMMARY

A method is described for measuring directly the volumetric or area ratios of components of tissues or organs under any conditions, where the various components are clearly distinguishable visually.

Tests of the method showing its degree of accuracy when applied to the analysis of fixed and stained preparations are presented.

Depolymerases for Yeast and for Thymus Nucleic Acids in Normal and Neoplastic Tissues

By JESSE P. GREENSTEIN, *senior biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

At least two kinds of nucleic acid exist in both animal and plant tissues. One of these contains *d*-ribose as the sugar component, the other contains *d*-2-desoxyribose. The most frequently studied representative of the ribosenucleic acids is that derived from yeast; the most frequently studied desoxyribosenucleic acid is that obtained from the calf thymus. Both yeast and thymus nucleic acids occur in a polymerized form in which the tetranucleotide structure is repeated many times to form a compound of high molecular weight estimated to be about 2×10^4 for the former (*1*), and about 1×10^6 for the latter (*2*). In this form, the nucleic acids possess specific physical properties. The yeast nucleic acid is relatively insoluble in acids, and solutions of the sodium salt of thymus nucleic acid show intense streaming birefringence and structural viscosity. When the nucleic acids are treated with tissue enzyme preparations from various sources, these properties are progressively diminished (*3, 6*). When the proper conditions are chosen, no further alteration in the nucleic acids occurs, e. g., splitting off of nucleotides or inorganic phosphorus. Such conditions include the maintenance of a neutral pH, a relatively short period of digestion, and in certain cases a heating of the enzyme preparation. Inasmuch as no inorganic phosphorus appears and no nucleotides are split off under the conditions chosen, the change in the physical properties of the nucleic acids by the enzyme preparations has been interpreted as a depolymerization of the tetranucleotide aggregates of high molecular weight into similar aggregates of lower molecular weight (*4, 6*). In the case of yeast nucleic acid the translational diffusion constant has been found to increase (*7*), and from digests of thymus nucleic acid tetranucleotides have actually been isolated (*8*).

The depolymerase for yeast nucleic acid, hereafter referred to as ribonucleodepolymerase, or RD, is heat-stable (*3, 4*) and hence distinct from the heat-labile (*5, 6*) depolymerase for thymus nucleic acid, hereafter referred to as desoxyribonucleodepolymerase, or DRD. Furthermore, the isolated and crystalline preparations of RD do not effect any enzymatic depolymerization of thymus nucleic acid (*7, 9*). It appears that each of the two kinds of nucleic acid requires a distinctly different enzyme system for its depolymerization.

As part of a general study of the changes in activity of certain enzymes which occur when normal tissues become neoplastic, it was observed that the DRD activity of normal mouse lymph nodes was the highest for any of the tissues studied (*10*). However, the activity of this enzyme in a transplanted mouse lymphoma was very low. Comparison of the DRD activity in normal liver and in transplanted hepatomas of the rat and of the mouse revealed that the normal and neoplastic tissues in the former species were very similar, but that the neoplasms were generally more active than normal in the latter species

(*10*). For further comparison with growing tissues, it was observed that fetal rabbit liver and regenerating rat liver had the same activity as adult rabbit liver and normal rat liver, respectively. Does RD activity change or remain constant under the same comparative conditions found for DRD? The investigation of this question forms the basis of the present paper.

EXPERIMENTAL PROCEDURE

Tissues

The mouse lymphomas studied, 72942 and Y103, have been described (*11*). The activity of these tissues was compared with that of normal mouse lymph nodes. Other tissues investigated included mouse pancreas, spleen, thymus, muscle, and kidney, normal rat, rabbit, and mouse livers, fetal rabbit livers (17 days old), regenerating rat liver (taken 48 hours after excision of 60–70 percent of the liver), transplanted hepatoma 31 in rats (*12*), and the transplanted hepatomas 98/15 (*13*), and 7A/77 (*14*) in mice. Further comparisons were made between the spleens of normal rats and of rats of the same age and size bearing the transplanted hepatoma 31. The spleens of the latter animals were about twice the weight and size of those of the former. The tissues in every case were removed immediately after death of the animal (generally by decapitation), and the necrotic areas dissected from the tumors and discarded. The tissues were ground in a mortar with sand and extracted with distilled water at 4° C. for 24 hours. The mixtures were centrifuged and the supernatant solutions used for the enzymatic determinations. All the determinations for RD activity were performed on extracts which had been adjusted with distilled water in each case to 0.4 mg. total nitrogen per cubic centimeter.

The lymphomas and the regenerating liver and mouse hepatomas were donated for these studies by Drs. Dalton and Eschenbrenner.

Enzyme Activity

The activity of DRD in extracts of the mouse pancreas, kidney, spleen, thymus, and muscle, and of the mouse lymphoma Y103 has not been previously reported. The activity of the enzyme in such extracts at a concentration of 1.23 mg. nitrogen per cubic centimeter was therefore determined exactly as described earlier (*6*, *15*). The method involved the measurement of the progressive decrease in the viscosity of mixtures of the tissue extracts with sodium thymonucleate at 30° C. and at 16.0-cm. water external pressure.

The activity of RD was determined as follows: To 1.0 cc. of the extract were added 1.0 cc. of a veronal acetate buffer at pH 7.0 and 1.0 cc. of a 3.0-percent solution of purified yeast sodium nucleate. The latter was purified by two acid precipitations from alkaline solutions of the nucleic acid. Control solutions, one containing water in place of the extract and the other containing water in place of the nucleate, were run simultaneously. After the designated period of incubation at 37° C., the tubes were chilled in ice water, and 1.0 cc. 1N hydrochloric acid was added with shaking to each. The tubes were allowed to remain in the ice water for 15 minutes and were then centrifuged. The supernatant solutions were digested with concentrated sulfuric acid and peroxide and analyzed for phosphorus by the Fiske-Subbarow method. The periods of incubation were set at 2 and 5 hours. The zero reading was taken by adding the hydrochloric acid immediately to the original mixture. The total phosphorus found in the nucleic acid control was the same after 5 hours of incubation as at the beginning

TABLE 1.—*The depolymerases for yeast and for thymus nucleic acids in normal and in neoplastic tissues*

Tissue [1]	Activity of ribonucleodepolymerase (RD)			Activity of desoxyribonucleodepolymerase (DRD)[2]
	Period of incubation	Total phosphorus soluble after addition of hydrochloric acid	Increase in total acid-soluble phosphorus	
	Hours	*Milligrams*	*Milligrams*	
Mouse thymus [3]	0	0.33		3.1
	2	.45	0.12	
	5	.63	.30	
Mouse pancreas	0	.32		5.4
	2	1.10	.78	
	5	1.10	.78	
Mouse lymph nodes	0	.31		[4] 25
	2	.77	.46	
	5	1.00	.69	
Mouse spleen	0	.32		16.1
	2	.60	.28	
	5	.91	.59	
Mouse liver	0	.32		14.3
	2	.44	.12	
	5	.62	.30	
Mouse kidney	0	.32		10.2
	2	.40	.08	
	5	.56	.24	
Mouse muscle [5]	0	.31		12.0
	2	.38	.07	
	5	.53	.22	
Lymphoma 72942 in mice	0	.33		3.9
	2	.50	.17	
	5	.61	.28	
Lymphoma Y103 in mice	0	.32		4.2
	2	.43	.11	
	5	.51	.19	
Hepatoma 98/15 in mice	0	.33		20.6
	2	.63	.30	
	5	.94	.61	
Hepatoma 7A/77 in mice	0	.33		15.4
	2	.62	.29	
	5	.92	.59	
Rat liver	0	.32		6.6
	2	.41	.09	
	5	.53	.21	
Regenerating rat liver (2 days after excision of lobes)	0	.33		6.3
	2	.41	.08	
	5	.55	.22	
Rat hepatoma 31	0	.32		5.3
	2	.39	.07	
	5	.53	.21	
Adult rabbit liver	0	.31		10.5
	2	.37	.06	
	5	.42	.11	
Fetal rabbit liver (17 days)	0	.31		10.4
	2	.37	.06	
	5	.42	.11	
Normal rat spleen	0	.33		1.7
	2	.62	.29	
	5	.77	.44	
Spleens of rats bearing hepatoma 31	0	.32		1.7
	2	.60	.28	
	5	.75	.43	

[1] Tissue extracts for each determination of RD contained 0.4 mg. nitrogen per cubic centimeter; for DRD activity each extract contained 1.23 mg. nitrogen per cubic centimeter.
[2] Calculated as before (*6*, *15*).
[3] C3H mice 2.5 months old.
[4] Probably still greater than value given (*15*).
[5] Skeletal muscle from hind legs.

and was practically identical with that found in the mixture of extract, buffer, and nucleate at the so-called zero time mark. The amount of protein in the extract was evidently too small to interfere with the precipitation by hydrochloric acid of the nucleate. The amount of phosphorus in the tissue extract control was too small, in every case studied, to measure and hence was neglected. It was for these several reasons that as low a concentration of 0.4 mg. nitrogen per cubic centimeter of the

extracts had been chosen. Readings were made in duplicate. These varied by no more than 5 percent. Duplicate samples of tissues gave readings that varied by no more than 10 percent.

The extent of RD activity for each tissue is given in terms of the increase in total acid-soluble phosphorus. Data for DRD at 30° C. are given for comparison and except for the cases of the normal mouse pancreas, kidney, spleen, thymus, muscle, and lymphoma Y103 are taken from an earlier report (*15*). The averaged data for all the readings are given in table 1.

It is evident that the greatest RD activity is found in the mouse pancreas and lymph nodes. Indeed, the activity in the former tissue apparently reaches a maximum value within 2 hours of incubation. The activity of the lymphomas is decidedly less than that of the normal nodes. The transplanted rat hepatoma has the same activity as that of the normal rat liver but that of the transplanted mouse hepatomas is distinctly greater than that of the normal mouse liver. Comparison with the relative activity of DRD in the normal tissues and the neoplasms which arise therefrom reveals the striking fact that exactly the same conclusions can be drawn in the case of both enzymes. When the normal tissue becomes neoplastic, both enzymes are altered in activity in the same direction.

A further striking extension of this observation is seen in the comparison of the activity of both RD and DRD in the following pairs of tissues: regenerating and normal rat liver, fetal and adult rabbit liver, and the enlarged spleens of tumor-bearing rats and the normal spleens of rats. The activity of each enzyme is practically identical in each pair of the tissues cited. The normal change of fetal into adult hepatic tissue or the normal regeneration of rat hepatic tissue is not accompanied by any observable variation in the activity of either enzyme.

Dialysis Experiments

In order to observe whether the effect of the tissue extracts as used is really a depolymerization of tetranucleotides of high molecular weight into tetranucleotides of lower molecular weight and not in addition a further splitting to mononucleotides and possibly inorganic phosphorus, the following experiments were made: (1) The determination of inorganic phosphorus in the digests; and (2) the determination of dialyzable organic phosphorus in the digests.

In no case investigated was there any evidence whatever, up to 4 hours' incubation of the tissue extracts with sodium thymonucleate (*6*) or up to 5 hours' incubation of the extracts with yeast nucleate, that any inorganic phosphorus was released from either nucleate. The dialysis experiments likewise revealed no formation of dialyzable phosphorus, whether organic or inorganic. The latter experiments were set up as follows: After 2 hours' digestion at 37°, the mixtures together with the controls were separately placed in cellophane bags and dialyzed against a large volume of distilled water for 3 hours. At the end of this time the outside fluids were concentrated and tested for inorganic phosphorus by direct analysis and for organic phosphorus after digestion with sulfuric acid. The negative results for dialyzable organic phosphorus, particularly in the case of the lymph node extracts were quite striking, perhaps even unexpected. Thus, the mixture of 3.0 cc. of 1.0 percent sodium thymonucleate and an equal volume of water had a value of the relative viscosity of 51.23 at 30° C. and 16-cm. water external pressure; when the 3.0 cc. of water is replaced by an equal volume of aqueous extract of the mouse lymph nodes, the

value of the viscosity of the mixture under the same conditions drops to 1.3 within 10 minutes (*6*). When such a mixture is further kept under these conditions for nearly 2 hours longer, then dialyzed for 3 hours, and finally does not show the presence of dialyzable organic phosphorus, it may be concluded that under the conditions the nucleate molecules may disaggregate but that the process of disaggregation does not lead to the formation of molecules small enough to pass through cellophane. Very roughly this means that no molecules smaller than a tetranucleotide (molecular weight 1,400) could possibly have been produced.

The failure to observe any dialyzable organic phosphorus after incubation with the depolymerases is in agreement with the results of Schmidt and Levene (*4*) but not with those of Kunitz (*7*). The former investigators, like the writer, employed tissue extracts as the source of the enzymes, Kunitz on the other hand used a crystalline preparation of RD. Whether this difference explains the divergence in the results obtained cannot be decided at the present time.

DISCUSSION

Desoxyribosenucleic acid is observed only in the nucleus of cells and constitutes a considerable proportion of the chromatin or chromosomal structure. DRD activity appears also to be confined to the nucleus for it is absent in enucleated cells (*5*). Ribosenucleic acid has been isolated only from cytoplasmic components (*16*, *17*), and it is likely, therefore, that RD activity would be expected to be at least principally a cytoplasmic function. Such a differentiation of nuclear and cytoplasmic activities based on nucleic acid distribution and metabolism is in the main probably true, but the possibility of some overlapping of these funkions in both morphologic structures cannot be entirely excluded. Nevertheless, it is of interest that when normal hepatic or lymphatic tissue becomes neoplastic, both DRD and RD, or presumably nuclear and cytoplasmic functions, respectively, are altered in parallel fashion.

It has long been known that embryonic tissues of many kinds are rich in purine bases (*18*, *19*) and in pentose (*20*). Caspersson has shown that embryonic cells (*21*) and also adult secretory cells as in the pancreas (*22*) are rich in substances which absorb ultraviolet radiation at 2600A, and has suggested that tissues in which a rapid rate of protein synthesis occurs contain a large proportion of ribosenucleic acid in the cytoplasm. There appears to be little direct evidence for the presence of nucleic acid as such, but it does appear to be true that embryonic tissues are rich in the components of ribose nucleic acid. The RD as well as the DRD activity of fetal rabbit liver is practically identical with the respective activity of these enzymes in adult liver. In this connection it may be pointed out that of the enzymes so far compared in fetal and adult liver, all but RD, DRD, and amylase are considerably lower in the former than in the latter tissue (*10*). What has been referred to as amylase is most probably a mixture of phosphorylating enzymes (*23*). It would appear that the activity of the latter system together with those of the depolymerases for nucleic acids is maintained at the same level, per unit of hepatic tissue nitrogen, from at least the latter half of the fetal period through adult existence. The activity of other enzymes in the fetal liver, such as the oxidases, catalase, arginase, etc., is apparently not so fully advanced at this stage.

Regenerating rat liver was selected at a period of 48 hours following excision in order to obtain material with a high level of mitotic activity. However, like all other

enzymes studied in regenerating and in normal resting liver (*24*) the activity of RD and DRD is respectively equal in both kinds of tissue. The enlargement of the spleen in tumor-bearing rats also appears to be unaccompanied by any change in the activity of either depolymerase.

SUMMARY

The activity of ribonucleodepolymerase (RD) and of desoxyribonucleodepolymerase (DRD) was determined in mouse kidney and pancreas and in the following pairs of tissues: normal rat liver and transplanted hepatoma as well as regenerating rat liver, normal mouse liver and three different transplanted mouse hepatomas, adult rabbit liver and fetal rabbit liver, normal mouse lymph nodes and two different mouse lymphomas, and normal rat spleens and the enlarged spleens of tumor-bearing rats. The action of the enzymes studied was limited to a depolymerization of the nucleates, for in neither case were dialyzable products obtained.

The activity of both enzymes was lower in the lymphomas than in the normal nodes, about the same in the rat hepatoma and in the normal and regenerating rat liver, distinctly higher in the mouse hepatomas than in normal mouse liver, and practically identical in adult and fetal liver and in the normal and enlarged spleens. Where one enzyme changes, the other likewise changes in the same direction; where one enzyme remains constant so too does the other. The parallel behavior of both RD and DRD is striking. These enzymes, moreover, as well as the carbohydrate phosphorylases, are the only enzymes the activity of which is as great in fetal as in adult liver.

The highest RD activity was observed in the pancreas and in the lymph nodes of mice in this order. The highest DRD activity was observed in the lymph nodes. The mouse kidney activity for both enzymes was somewhat less than that for the liver of the species.

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Note on the Colloid Osmotic Pressure of the Serums of Rats Bearing the Transplanted Jensen Sarcoma

By JESSE P. GREENSTEIN, *senior biochemist*, and J. W. THOMPSON, *pharmacologist*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

As part of a general program designed to study the possible systemic effects elicited in tumor-bearing animals by the presence of the tumor (*1*), the colloid osmotic pressure of the serums of Buffalo rats inoculated with the Jensen sarcoma was investigated. Forty-two rats of the same sex (female) and of approximately the same age and weight were inoculated with the tumor. At selected intervals, three rats were killed by decapitation, the blood was collected from each, and the serums were separated from the clots by centrifugation. The method of measuring the osmotic pressure has been described (*2*). Up to about the first week after inoculation the tumors were not visible. Thereafter, an effort was made to select animals with approximately the same sized tumors at each time interval. The intervals were selected up to 3 weeks following inoculation at which time the animals begin to die off. Since the tumors were inoculated intramuscularly in the leg of these animals, it was impossible to dissect out the entire growth for the determination of the tumor mass, and for this reason there had been no attempt at correlation of the observations made with the tumor mass itself. The temperature ranged from 23° to 24° C. The data are collected in the following tabulation:

Days following inoculation	*Colloid osmotic pressure* [1] *(in millimeters of water)*		
0 (control)	310	347	320
2	290	321	309
5	284	289	285
7	261	254	278
8	265	254	270
10	256	262	280
12	240	230	252
14	217	218	242
17	222	200	205
19	205	200	199
21	200	208	207

[1] Each reading refers to the equilibrium value (after 45 minutes of placing undiluted serum sample in osmometer) for the serum of a single animal. Three rats were used at each time interval.

It is clear that there is on the whole a progressive decrease in the colloid osmotic pressure of the serums of the animals as the tumors continue to grow. The over-all drop in 3 weeks after inoculation is about 30 percent. The decrease in the pressure is most probably to be attributed to a decrease in the protein concentration of the serum. The protein concentration was determined by adding 1.0 cc. of 5 percent trichloracetic acid to 0.5 cc. of serum; after centrifugation, decantation, and washing with dilute precipitant, the residue was tested for nitrogen. It was assumed that the average nitrogen percentage for the serum proteins was 16.0. Four samples

of normal rat serums (from as many animals) yielded 8.4, 8.0, 9.3, and 8.7 percent protein; four samples of the serums of tumor-bearing rats (3 weeks after inoculation) yielded 7.0, 7.6, 6.5, and 6.2 percent protein. The difference here is about 15 percent. There need not necessarily be a linear proportionality between protein concentration and osmotic pressure in view of the possibility of different states of aggregation of the proteins in the serums of the normal and of the tumor-bearing animals.

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Serologic and Anaphylactic Reactions of the Cathepsins of Normal and Neoplastic Tissues

By MARY E. MAVER, *senior biochemist*, and MORRIS K. BARRETT, *research fellow*,[1] *National Cancer Institute, National Institute of Health, United States Public Health Service*

Recent work (*1*) showed that the cathepsins, or endocellular proteinases, of some organs and tumors were able to hydrolyze certain synthetic peptides and not others. Thus, the cathepsin from rat spleen hydrolyzed benzoylglycylglycine while the cathepsins from Jensen rat sarcoma and rat kidney did not. Cathepsins from rat kidney and liver hydrolyzed chloracetyl-*l*-tyrosine while those from Jensen sarcoma and rat spleen did not. The differences in the specific activities of the enzymes may be related to the growth rate of the tissues from which the cathepsins were obtained, since they are probably responsible for the synthesis of protein, and to the different physiologic functions of the tissues. The interesting question arises whether an altered endocellular proteinase might be a factor in the more rapid growth of a neoplasm as compared with its tissue of origin.

Insofar as elementary chemical analyses have progressed, they have not shown essential differences in the catheptic proteins from Jensen rat sarcoma, hepatoma 31, rat liver, spleen, and kidney. Since the cathepsins were found to be antigenic, their serologic and anaphylactic specificity was studied in order to learn whether their protein structures were identical, closely related, or different.

[1] *Now Major, M. C., A. U. S.*

EXPERIMENTAL PROCEDURE

The cathepsins used were lyophilized preparations separated from the frozen, pooled tissue [2] by the method previously described (*2*). Hepatoma 31 was induced originally in Osborne-Mendel strain rats by feeding them *p*-dimethylaminoazobenzene (*3*), and subcutaneous transplants of a primary hepatoma were used in these experiments. Those rats in which the hepatoma 31 transplants did not grow or in which there was regression after initial growth, were called resistant rats. The Jensen sarcoma tissue was obtained from intramuscular transplants grown in a strain of rats which originally came from Buffalo and which have been pen-bred in this laboratory for about 20 years. These rats are designated N. C. I. rats.

For the precipitin and complement-fixation tests, antiserums were prepared by intravenous injections into rabbits, weighing 1.5 kg., on 4 alternate days with one quantity of cathepsin and then doubling the quantity injected on 4 alternate days after a week's interval. In this way one series of rabbits received intravenous injections of 10 and 20 mg. per kilogram of body weight, of each of the cathepsins from hepatoma 31, normal Osborne-Mendel rat liver, and resistant rat liver.

[2] *Dr. J. W. Thompson, of this Institute, furnished the tumor tissues.*

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In another series three rabbits each received four injections of 3.75 mg. and four injections of 7.5 mg., per kilogram of body weight, of the cathepsins from hepatoma 31, Jensen rat sarcoma, and from the livers of normal Osborne-Mendel rats. Eleven days after the last injection the rabbits were bled from the heart. The titers of only the most potent of each type of serum are given.

The precipitin tests were made in the usual way by layering 0.2 cc. of a saline solution of an enzyme upon 0.2 cc. of the antiserum. The width of the ring formed at the boundary and the amount of precipitate which settled upon centrifugation were used to determine the potency or degree of specificity of the precipitins in the serums.

The titrated hemolytic system for the complement-fixation tests was supplied by Dr. Ida Bengtson and Mr. Turner, of the National Institute of Health. The hemolysin was diluted to contain 2 units in 0.2 cc., and the complement diluted to contain 1⅓ units in 0.2 cc. The antiserums were inactivated by heating at 56° C. for 1 hour immediately before use. Mixtures of 0.2 cc. each of diluted serum, complement, and enzyme solution were kept at 37° for 1 hour. Then 0.4 cc. of a mixture of equal volumes of 2-percent washed sheep cells and hemolysin were added. The sheep cells had been sensitized by contact with the hemolysin for 10 minutes before they were used. After 1 hour in the water bath at 37°, the cells were allowed to settle in the cold room, and readings of the degree of hemolysis were made the following morning. Both the serum and the cathepsins were tested for anticomplementary action.

Most of the anaphylactic tests were made on guinea pigs of family 2, weighing 200 to 300 gm. This inbred strain of guinea pigs was obtained originally from the Bureau of Animal Industry, United States Department of Agriculture (*4*). The sensitizing dose was injected intraperitoneally. Three weeks later the shocking dose was injected into a foot vein. The second series of anaphylactic tests were made with stock guinea pigs.

RESULTS

In order to detect reactions from contaminating proteins which might be common to the catheptic preparations from different sources, a series of rabbits were sensitized with small amounts of cathepsins, 3.75 and 7.5 mg. per kilogram of body weight, as well as with larger amounts, 10 and 20 mg. The marked similarity of the relative titers obtained with the serums of high and low antibody concentration attest the purity of the catheptic proteins. The different cathepsin preparations from the same tissue source, having the same proteolytic activities, also gave the same titers when used in the precipitin tests.

Landsteiner's explanation (*5, p. 23*) of the serologic specificity of proteins, based upon the protein theories of Sörensen and Svedberg, is that "the specificity of proteins is an attribute of the unit, that is, the smallest component which cannot reversibly be split." Accordingly, the highest titers, or most dilute antigen-antibody responses, are obtained when an antiserum reacts with its homologous antigen where the two proteins used have identical unit composition. On this basis lower titers would result when a different protein was used for sensitization than was used for reaction with the antibody since some or all of the units or groups of the proteins would be different. The varying precipitin titers of the cathepsins with the antiserums furnished a serologic differentiation of the catheptic proteins from different tissues. Thus, the titer of the homologous antigen was 1 : 10,000,000 in the case of the anti-

TABLE 1.—*Precipitin tests with antiserums to cathepsins using different concentrations of cathepsins as antigens and serums diluted 1:2*

Source of antigens	Antiserum to hepatoma 31 cathepsin					Antiserum to normal rat liver cathepsin					Antiserum to resistant rat liver cathepsin				
	Dilutions of antigen					Dilutions of antigen					Dilutions of antigen				
	1:1,000 [1]	1:10,000	1:100,000	1:1,000,000	1:10,000,000	1:1,000	1:10,000	1:100,000	1:1,000,000	1:10,000,000	1:1,000	1:10,000	1:100,000	1:1,000,000	1:10,000,000
Hepatoma 31:															
Preparation 1	+++++	+++	+	+	+	++	+	0	0	0	++	+	0	0	0
Preparation 2	+++++	++	+	+	+	+++	+	+	0	0	+++	+	0	0	0
Preparation 3	++++	++	+	+	+	+++	++	0	0	0	++	+	0	0	0
Jensen sarcoma:															
Preparation 37	++	+	+	+	0	+++	+	0	0	0	+	?	0	0	0
Preparation 38	++	+	+	+	0	+++	+	0	0	0					
Preparation 40	++	+	+	+	0	++	+	0	0	0	+	+	0	0	0
Normal rat liver:															
Osborne-Mendel strain	{+++ +++}	+	0	0	0	{++++ +++}	++	+	+	+	+++	+	+	+	0
N. C. I. stock	+++	+	0	0	0	++++	++	+	+	+	++	+	+	?	0
Resistant rat liver:															
Preparation 28	+	+	0	0	0	++++	++	+	+	+	+++	++	+	+	0
Preparation 29	{					+++	++	+	+	0	++++ +++	+++	++ +	+	0
Normal rat kidney	+	+	0	0	0	+	+	0	0	0	++	+	?	0	0
Normal rat spleen	+	0	0	0	0	+	+	0	0	0	+	0	0	0	0
Spleens of rats bearing Jensen sarcoma	+	+?	0	0	0	+	+	0	0	0	+	+	0	0	0
Beef spleen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rat muscle albumin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hepatoma 31 albumin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rat liver albumin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1] 1:1,000 is equivalent to 1 mg. dry weight of antigen per cubic centimeter.

TABLE 2.—*Precipitin tests with different dilutions of antiserums to cathepsins using cathepsins in a 1:1,000 concentration (1 mg. per cubic centimeter) as antigens*

Source of cathepsins	Antiserum to hepatoma 31 cathepsin										Antiserum to normal rat liver cathepsin										Antiserum to resistant rat liver cathepsin									
	Dilution of serum										Dilution of serum										Dilution of serum									
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024
Hepatoma 31	+	+	+	+	+	+	+	+	+	?	+	+	+	+	0	0	0	0	0	0										
Preparation 2	+	+	+	+	+	+	+				+	+	+	+	0	0	0	0	0	0	+	+	+	+	+	+	+	+		
Preparation 3	+	+									+	+									+	+	+	+	+	+	+	+		
Normal liver:																														
Osborne-Mendel rat	+	+	+	+	+	0	0	0	0	0	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+		
Liver of resistant rats																					+	+	+	+	+	+	+	+		

serum to hepatoma 31, while the titer with the heterologous antigen, normal liver cathepsin, was 1:10,000 (table 1). The antiserum to hepatoma 31 cathepsin reacted in a dilution of 1: 512 with hepatoma 31 cathepsin and in a dilution of 1 : 32 with normal liver cathepsin (table 2). These results showed that the protein of the normal liver cathepsin was not identical with the protein of hepatoma 31 cathepsin, but that they may have had some common protein units which induced an antigen-antibody response in the lower dilutions.

The high precipitin titer of the Jensen sarcoma cathepsin with the antiserum to hepatoma 31 cathepsin indicated that the proteolytic enzymes of the two neoplastic rat tissues have more closely related chemical structures than have hepatoma 31 and normal liver cathepsins (table 1). This result was confirmed when the antiserum to Jensen sarcoma cathepsin was titrated with hepatoma 31 cathepsin and the normal liver cathepsin (table 3). The close relationship of the cathepsins from the two neoplastic rat tissues was demonstrated again in the complement-fixation tests (table 4). The complement was fixed in higher dilutions of the antiserum to hepatoma 31 cathepsin with Jensen sarcoma cathepsin than with normal liver cathepsin as antigen. It is interesting that the complement-fixation tests showed a slightly closer relationship of the liver cathepsin to hepatoma 31 cathepsin than to the sarcoma cathepsin.

There was no evidence in the precipitin tests of a difference in the cathepsins of the livers of the normal or resistant Osborne-Mendel rats and the N. C. I. strain which was used for transplantation of the Jensen sarcomas. The reactions of the rat kidney and spleen cathepsins with the antiserums to the normal liver and tumor cathepsins occurred only in the lower dilutions of antigen, and therefore these enzymes may

TABLE 3.—*Precipitin tests with antiserums to cathepsins using different concentrations of cathepsins as antigens and serums diluted 1:2*

Source of cathepsins	Antiserum to hepatoma 31 cathepsin					Antiserum to Jensen sarcoma cathepsin					Antiserum to normal liver cathepsin				
	Dilutions of antigen					Dilutions of antigen					Dilutions of antigen				
	1:1,000	1:5,000	1:10,000	1:50,000	1:100,000	1:1,000	1:5,000	1:10,000	1:50,000	1:100,000	1:1,000	1:5,000	1:10,000	1:50,000	1:100,000
Hepatoma 31:															
Preparation 1	+++	+++	++	+	0						++	+	+	0	0
											++	+	0	0	0
											+++	+	+	0	0
Preparation 2											++	+	0	0	0
Preparation 3	+++++	+++	++	+	+	+++	++	++	+	+	++	++	+	0	0
	++++	+++	++	+	0	+++	++	++	+	0	++	+	+	0	0
Jensen sarcoma:															
Preparation 37	++	+	+	+	0										
	++	+	+	+	0										
Preparation 39	++	+	+	0	0	++	++	+	+	+	+	+	0	0	0
						++	++	+	+	+	+	0	0	0	0
						++	++	+	+	0	+	0	0	0	0
Preparation 40	+++	++	+	+	0	++++	+++	++	+	0	++	+	0	0	0
	+++	+	+	0	0	+++	+++	+	+	0	+	+	0	0	0
Normal liver:															
Osborne-Mendel strain 33	++	++	+	+	0	++	++	+	0	0	+++	++	+	+	0
	+++	++	+	0	0	++	++	+	0	0	+++	+	+	+	0
						+	+	0	0	0	++	+	+	+	0
N. C. I. strain 30	+++	+	+	0	0	+	+	0	0	0	+	+	+	?	0
Resistant rat liver:															
Preparation 29	++	+	+	0	0						+++	+	+	0	0
Spleens of rats bearing Jensen sarcoma	+	+	0	0	0	+?	0	0	0	0	+	?	0	0	0
						+	0	0	0	0	+	0	0	0	0
Normal rat kidney:															
Preparation 5	++	+	+	0	0	++	+	0	0	0	+	+	+	0	0
	++	+	0	0	0	+	+	0	0	0	+	+	+	0	0

TABLE 4.—*Complement-fixation test with antiserums to cathepsins from neoplastic rat tissues using cathepsins as antigens in a dilution of 1 : 1,000*

Source of cathepsin	Antiserum to hepatoma 31 cathepsin II								Antiserum to Jensen sarcoma cathepsin 40							
	Serum dilutions								Serum dilutions							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Hepatoma 31:																
Preparation 2	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	0	0
Preparation 3	+++	+++	+++	+++	+++	+++	+++	0	+++	+++	+++	+++	+++	+++	0	0
Normal liver:																
Preparation 33	+++	+++	+++	+++	++	0	0	0	+++	+++	+++	++	0	0	0	0
Preparation 31	+++	+++	+++	+++	+	0	0	0	+++	+++	+++		0	0	0	0
Jensen sarcoma:																
Preparation 39	+++	+++	+++	+++	+++	+++	0	0	+++	+++	+++	+++	+++	+++	+++	0
Preparation 40	+++	+++	+++	+++	+++	+++	++	0	+++	+++	+++	+++	+++	+++	++	0

0, no fixation (complete hemolysis); ++++, complete fixation (no hemolysis).

have somewhat different chemical structures.

Definite species specificity was shown by the failure of the beef spleen cathepsin to react with any of the antiserums to rat cathepsins. The beef spleen cathepsin was the most active cathepsin prepared, as shown by the hydrolysis of hemoglobin, and its failure to react in these serologic tests may indicate that the protein structure which is responsible for the hydrolysis of proteins is different in different species.

Other water-soluble rat protein preparations, the albumins from striated muscle, normal liver, and hepatoma 31, gave negative precipitin tests with the antiserums to rat tissue cathepsins, which clearly differentiated these proteins from the catheptic proteins.

The specific antigen-antibody response of the anaphylactic reaction in guinea pigs was also used to determine the chemical relationship of the cathepsins. When the family 2 guinea pigs were sensitized with varying amounts of hepatoma cathepsin, much more normal liver cathepsin than hepatoma cathepsin was required to induce fatal anaphylactic shock (table 5). Similar results were obtained with the stock guinea pigs (table 6). Animals which were sensitized with 0.5 mg. of hepatoma cathepsin per kilogram of body weight died in shock with 5 mg. of hepatoma 31 cathepsin or 12 mg. of Jensen sarcoma cathepsin but showed no symptoms of shock with 30 mg. of resistant-rat-liver cathepsin. Animals sensitized with 0.5 mg. of normal liver cathepsin per kilogram of body weight died in shock with 6 mg. of normal liver cathepsin but showed no symptoms of shock with 30 mg. of hepatoma 31 cathepsin, and only slight symptoms with 20 mg. of Jensen sarcoma. Therefore, the same relative differences and similarities of the chemical composi-

TABLE 5.—*Anaphylactic tests with family 2 guinea pigs*

Sensitizing cathepsin		Number of animals	Cathepsin used for shocking		Results of shocking dose
Source	Dose per kg. body wt.		Source	Dose per kg. body wt.	
	Milligrams			*Milligrams*	
Hepatoma 31	1	1	Hepatoma 31	5	Fatal shock.
	1	1	do	4	Do.
	1	1	Normal liver	4	No symptoms.
	1	2	Hepatoma 31	3	Fatal shock.
	1	1	do	2	Survived shock.
	.5	2	do	4	Do.
	.5	2	do	5	Fatal shock.
	.5	2	Normal liver	5	Survived shock.
	.25	1	Hepatoma 31	6	Survived severe shock.
	.25	1	do	10	Fatal shock.
	.25	1	Normal liver	10	Survived shock.
	.25	1	do	20	Fatal shock.
	.10	1	Hepatoma 31	7.5	Survived shock.
	.10	2	do	10	Fatal shock.
	.10	1	Normal liver	15	Survived.
	.10	1	do	25	Do.

TABLE 6.—*Anaphylactic tests with stock guinea pigs*

Sensitizing cathepsin		Number of animals	Cathepsin used for shocking		Results of shocking dose
Source	Dose per kg. body wt.		Source	Dose per kg. body wt.	
Hepatoma 31:	*Milligrams*			*Milligrams*	
Preparation 2	0.5	1	Hepatoma 31	5	Fatal shock in 2 minutes.
Do	.5	1	do	5	Do.
Do	.5	1	Normal liver	10	No symptoms.
Do	.5	1	Resistant rat liver	20	Do.
Do	.5	1	do	30	Do.
Do	.5	1	Jensen sarcoma	10	Slight symptoms.
Do	.5	1	do	12	Died in shock after 2 hours.
Do	.5	1	do	20	Fatal shock in 2 minutes.
Normal rat liver:					
Preparation 33	.5	2	Normal liver	6	Severe symptoms.
Do	.5	1	Resistant rat liver	10	Fatal shock.
Do	.5	2	do	5	Do.
Do	.5	2	Hepatoma 31	30	No symptoms.
Do	.5	2	Jensen sarcoma	20	Very slight symptoms.

tion of the cathepsins were demonstrated by anaphylaxis.

DISCUSSION

The specific antigen-antibody reaction has often been used to differentiate extracts of normal and neoplastic tissues. Sievers (*6*) reported the detection by means of the precipitin test of specific structures in malignant human tissues which could be sharply differentiated from species-specific, organ-specific, and group-specific characteristics. Reichner (*7*) was able to differentiate normal brain tissue from infiltrating glioma tissue by the complement-fixation test. This test was also used by Witebsky and Morelli (*8*), Morelli (*9*), and Hirszfeld, Halber, and Laskowski (*10*) to differentiate extracts of normal and neoplastic tissues. Although these authors were unable to obtain antiserums which reacted exclusively with extracts of neoplastic tissues, they did obtain quantitative differences in the titers which were made more significant when proteins common to both tissues were removed by adsorption.

Mann and Welker (*11*, *12*, *13*) prepared antiserums to an emulsion of the water-insoluble proteins of neoplasms which had been adsorbed on aluminum cream. Autolysates of the emulsions were used in

the precipitin tests. The antiserum to carcinoma of the stomach reacted positively to autolysates of carcinoma of the stomach in four of seven cases, and the antiserum to carcinoma of the cervix reacted positively to autolysates of carcinoma of the cervix in seven of eight cases. The autolysates of normal tissues gave negative results. Species specificity was shown where antiserums to rat carcinoma 256 and rat sarcoma 10 reacted with autolysates of these tumors but did not react with autolysates of carcinoma of the human breast or rectum.

Kidd (*14*, *15*) and Kidd and Friedewald (*16*, *17*) have described a distinctive substance associated with the Brown-Pearce rabbit carcinoma which is separated by centrifugation and which reacts with the serum of rabbits bearing Brown-Pearce tumors and also with the serum of normal rabbits. Barrett (*18*) demonstrated an immunologic difference between the extracts of chicken tumor and similar material from normal tissues, using the active particulate fraction of chicken tumor isolated by the method of differential sedimentation.

Few, if any, serologic investigations with the purified protein fractions of neoplastic tissues have been reported, although many closely related proteins of normal tissues have been studied. Thus, Hektoen, Robscheit-Robbins, and Whipple (*19*) have shown that hemoglobin from blood and from muscle are different, and Hektoen and Welker (*20*) have demonstrated two immunologically distinct crystalline Bence-Jones proteins.

It is not yet known whether the cathepsin of the primary hepatoma 31 also differs from the cathepsin of the normal rat liver. In this research the catheptic protein of transplanted hepatoma 31 has been found to be different from that of the normal liver from which the hepatoma originated. Recently the manner in which these two enzymes hydrolyze glutathione has been shown to differ (*21*). This change may be related to the fact that the hepatoma cell no longer performs all the functions of a liver cell and has acquired a more rapid growth rate. That these two enzymes are not entirely different, as shown by the cross precipitin reactions at the lower titers, seems reasonable since both enzymes hydrolyze many of the same proteins and synthetic peptides. The similarity of hepatoma 31 cathepsin and Jensen sarcoma cathepsin, as indicated by the high precipitin titers in cross reactions, suggests again that the cathepsin may be a factor in the growth of the neoplasms.

SUMMARY

The results of precipitin, complement-fixation, and anaphylactic tests indicated that the cathepsins of normal rat liver and transplanted hepatoma 31 are different proteins with some common protein groups.

These tests also showed that the cathepsins of Jensen sarcoma and transplanted hepatoma 31 are more closely related in chemical structure than are the cathepsins of hepatoma 31 and normal liver.

Rat kidney and spleen cathepsin reacted with low titers to the antiserum to the cathepsins of normal liver, hepatoma 31, and Jensen sarcoma.

Anaphylactic tests with guinea pigs confirmed the results of the precipitin and complement-fixation tests. Much more normal liver cathepsin than hepatoma cathepsin was needed to produce shock in an animal sensitized with hepatoma cathepsin.

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Accuracy and Reproducibility in the Induction of Tumors With Ultraviolet Radiation

By HAROLD F. BLUM,[1] formerly *senior pharmacologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

In previous papers on the induction of tumors with ultraviolet radiation, the limits of accuracy and the relationship between dose and response have been considered (*1*, *2*). Further data have been accumulated in the course of subsequent experiments which permit more exact evaluation of these factors in the present paper. It must be pointed out that the values arrived at herein apply only to a given set of experimental conditions, i. e., the exposure of male strain A mice to ultraviolet radiation of high intensity having a particular spectral distribution. No fundamental differences are to be anticipated if other experimental conditions are employed, but minor quantitative divergences are to be expected. To obtain corresponding accuracy and reproducibility numerous physical factors such as intensity and wavelength of the radiation must be properly controlled, and biologic factors such as individual variation and proportion of tumor types must be evaluated. These and other factors have been considered in papers already presented in this journal (*1*, *2*, *3*, *4*, *5*).

STATISTICAL TREATMENT OF INDIVIDUAL VARIATION

In the previous treatment of individual variation (*1*) groups consisting initially of 25 to 26 mice, and terminally of 15 to 26 mice were compared. Each of these groups had been subjected to continued repeated exposures to ultraviolet radiation, the dosage varying for different groups. While in a number of instances 2 or more groups had been exposed con-

[1] *Now principal biologist, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md.*

TABLE 1.—*Assembled data on tumor development*

Start of experiment	Groups	Animals in groups	Dose per exposure (Ergs/cm.^{2} x 10^7)	Statistics from direct calculation		Statistics obtained graphically		
				t_{50}	Standard deviation	t'_{50} (calculated)	Standard deviation	t'_{50} (drawn)
		Number		*Days*	*Log days*	*Days*	*Log days*	*Days*
April 1942	CM, CN	51	5.0	154.0	0.0850	150.3	0.0815	148.9
July 1940	B, F, N	63	3.3	155.1	.0811	152.4	.0894	153.1
May 1941	AR, AS, AT, AU	98	2.6	157.5	.0835	155.2	.0796	155.2
July 1940	C, G, O	63	2.0	158.5	.0788	154.8	.0772	154.5
January 1942	CH, CI	42	1.8	169.0	.0787	166.3	.0807	166.3
May 1942	CO, CP	44	1.8	167.5	.0817	162.9	.0864	164.1
March 1941	AA, AE, AI	70	1.6	172.5	.0870	169.8	.0821	169.8
March 1941	AB, AF, AJ	67	1.1	213.3	.0826	212.3	.0712	211.8
March 1941	AG, AK	42	.72	267.6	.0863	265.5	.0876	266.7
August 1941	BE, BF	47	[1] 2.0	141.4	.0727	141.3	.0796	141.3
August 1941	BG, BH	48	[1] .97	170.0	.0916	165.9	.0912	165.9
May 1942	CQ, CR	41	[2] 1.8	212.8	.0795	208.9	.0650	209.9

[1] Exposed 7 days per week, all other groups exposed 5 days per week (*1*).
[2] Low intensity groups (*6*).

currently to the same dosage the intensity of the radiation was not in all cases the same, and with limited information as to the influence of intensity it did not seem wise to combine such groups for statistical treatment. Subsequent examination of the intensity factor (*6*) shows that it is permissible to combine certain of these groups. Others have been added from subsequent experiments. Table 1 gives statistics determined for 12 such combined groups comprised terminally of from 41 to 98 animals. Each of these groups was formed by combining 2 to 4 smaller groups which were exposed concurrently, i. e., on the same days; the animals in the small groups were exposed simultaneously.

A wide variety of dosages are presented in table 1, and there is correspondingly wide variation of the time to 50-percent incidence of tumors. The latter value has been estimated in two ways. The first estimate is the antilogarithm of the mean of the logarithms of the individual development times,[2] which is designated by the symbol t_{50}. The logarithmic mean is used because of the nature of the distribution of the individual development times (*1*). The standard deviation for the logarithms of the individual development times has been calculated for each group. The mean of the standard deviations for the group is 0.0824 log days. This value is to be compared with the mean of the standard deviations observed for the smaller groups as analyzed in the earlier paper (*1*), 0.084 log days. The mean values for the standard deviation are in essential agreement, but the present value should be the more accurate.

The second estimate of the time to 50-percent tumor incidence is obtained graphically. For this purpose, the cumulative tumor incidence is plotted in probits (integrated probability units) against the logarithm of the individual development time. This yields a straight line (*1*). The point corresponding to 50-percent tumor incidence (5 probits) is obtained from this graph; the antilogarithm of the value so obtained is designated t'_{50}. Values of t'_{50} are given in table 1; all are slightly less than the corresponding values for t_{50}, the difference being expressed by $t'_{50}=t_{50}-1.6$. The mean value for the difference between t'_{50} and t_{50} obtained from the analysis of smaller groups (*1*) was virtually the same, but the deviation from the mean was much greater, as might be expected from the fact that smaller numbers were used. The slope of the line obtained by this method of plotting represents the reciprocal of the standard deviation. The reciprocal of this slope for the various groups is shown in table 1. The mean value, 0.0810 log days, is in essential agreement with the corresponding value of the standard deviation obtained directly. The values of t'_{50} (column 7) were obtained from the line calculated to best fit the experimental points. In making this calculation a line is first drawn by sight to secure a basis for properly weighting the points. In column 9, the values of t'_{50} obtained from these lines are given. These values all differ by less than 2 percent from the corresponding calculated values, and hence it appears that no useful increase in accuracy is obtained by making the calculation to obtain the best fitting line, provided the group consists of 40 animals or more. In drawing the lines the extreme points should be neglected for reasons discussed elsewhere (*1*).

It might be expected that t_{50} would be the more accurate value because it is based on the total number of animals which survive to the end of the experiment, and because it is not greatly affected by

[2] *The term "development time" is used to replace "induction time" employed in earlier papers. The reasons for this change have been discussed elsewhere* (7).

mortality from causes not connected with exposure to ultraviolet radiation. This was the position taken in reporting our earlier studies. However, t'_{50} has certain practical advantages (*1*), and the data assembled in table 1 indicate that it is just as accurate for experimental purposes when numbers of animals greater than 40 per group are used. In fact it seems probable that t'_{50} may be the more reliable value for the following reason: The individual development time is determined by inspecting the animals at regular intervals, and selecting those with tumors of greater than a given size (*4*). With this procedure there is a tendency to select late, that is when the tumor has grown somewhat beyond the standard size for selection. As a result the estimated individual development times are frequently too long, particularly if the animals are selected at relatively infrequent intervals. This should be reflected in the determination of t_{50}, tending to give high values. On the other hand, in determining t'_{50}, it makes little difference how infrequently the examinations are made, within obvious limits, since the points obtained determine a cumulative curve. It seems probable that the slightly greater values consistently found for t_{50} result in this way.

Whatever the cause of the divergence, the difference between the two values is small, and there seems every reason to use t'_{50} for comparative purposes simply on the basis of convenience. In the previous study with smaller groups it seemed wise to allow for a greater degree of variability when t'_{50} was used (*1*), but the uniformity of the differences between this value and t_{50} which are shown in table 1 would indicate that this is unnecessary, particularly when large numbers of animals are used.

The limits of significance in terms of numbers of animals used, assigned in table 2 of our previous study (*1*), are not essentially altered by the present analysis. The only modification of our previous position that need be made in this respect is that the same limits applied to t'_{50} are also applicable to t'_{50}, at least if numbers of animals greater than 40 per group are used.

DOSE-RESPONSE CURVE AND ITS REPRODUCIBILITY

In figure 1 are assembled the data in the form of dose-response curves, t'_{50} being plotted against dose of ultraviolet radiation per exposure. Besides the data from table 1, additional groups consisting of smaller numbers of animals and hence less accurate (groups A, BI, BJ, J, AN, AO, AP, and see (*1*)) have been included in constructing the graph. Groups CQ, CR, and others, which were exposed to radiation of low intensity (*6*), were not used.

The data for five exposures per week are the most extensive. These include the most accurately determined points and demonstrate their reproducibility nicely. The first column of table 1 gives starting dates for the experiments included therein, which range over approximately 2 years. If the corresponding points are identified on the graph (fig. 1) it will be seen that all fall along the drawn curve within the accuracy indicated. It is obvious that minor varia-

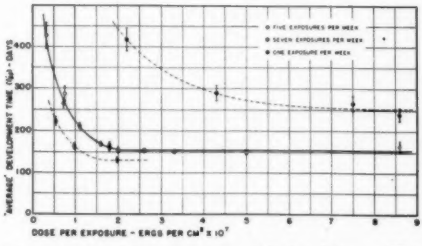

FIGURE 1.—Dose-response relationships. The vertical lines accompanying each point indicate the limit of error ($P=0.05$).

tions in experimental conditions such as seasonal fluctuations in temperature have had little or no effect. The reproducibility of the points is striking, particularly since reports of studies with carcinogenic chemicals indicate considerable variation between experiments carried out at different times but without known difference in the experimental technique (8). It seems likely that one factor which contributes to the reproducibility of studies with ultraviolet radiation is the large number of exposures which it is necessary to give to each group (7), for with repeated exposures minor fluctuations of experimental conditions or condition of the animals themselves might be expected to "average out." Experiments with chemical carcinogens, in contrast, usually involve one or a few applications of the agent, and such fluctuations may have much greater importance in determining the results.

The shape of the dose-response curve for five exposures per week substantiates the earlier finding (4) that there is a minimum time for tumor induction under a given schedule of exposures, which cannot be reduced by increasing the dose per exposure. In figure 1, dose per exposure is plotted instead of dose per week as in the graph previously presented (2), and hence the relative positions of the curves representing the three exposure intervals are different. This plotting emphasizes the fact that interval between exposures is, in a sense, more critical in determining development time than is dose per se. (See (7).)

In a recent paper Bain and Rusch (9) plot a selected portion of our earlier data (2) as total dose vs. log t_{50} and draw a straight line through the points. Neither the present data nor our earlier data yield a straight line when so plotted. The use of total dose in describing carcinogenic action is not justified in this type of experiment,

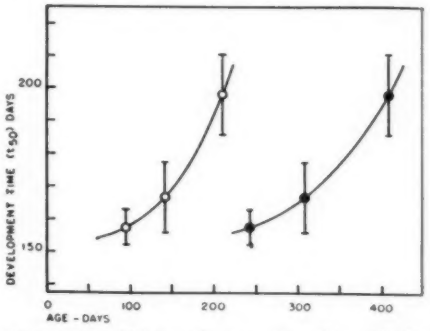

FIGURE 2.—Effect of age on t'_{50}. Circles represent age at first exposure; solid disks, age at t'_{50}. Vertical lines indicate limits of error ($P=0.05$).

since total dose is not independent of t_{50}. Inspection of figure 1 shows that with decreasing dose level t_{50} always increases. The meaning of Bain and Rusch's statement that small amounts of energy applied over a long period of time appeared "more efficient" for tumor production than larger doses given during shorter periods is not clear. (See (7).)

EFFECT OF AGE

The effect of age on the dose-response curve has been discussed elsewhere (2). The results of experiments bearing on this are shown in figure 2. Here, t'_{50} is plotted against the average group age at the first exposure in the first curve, and against the the age at t'_{50} in the second curve. A clear-cut increase in t'_{50} with age is indicated, although the points do not permit an accurate curve to be drawn. It appears, however, that t'_{50} varies less with age in young than in old animals. In the experiments on which figure 1 is based, the average group age has ranged from 2 to 3½ months at the outside. The first curve in figure 2, as drawn, indicates a variation in t'_{50} of about 3.5 percent over this range. Such a variation would not constitute a very important error in the experiments described herein. Had older animals been

used, however, a greater error might have been expected; and had animals of widely different ages been included in the same groups, the standard deviation for t'_{50} would have been greater.

SUMMARY

High reproducibility of results is obtained in experiments on the induction of tumors with ultraviolet radiation, if proper consideration is given to control of conditions and to statistical aspects.

The accuracy to be expected in making comparison of experimental groups is discussed, and additional information regarding the dose-response curve is presented.

The effect of age of the animals on tumor development is also considered.

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Chemical Treatment of Tumors. V. Isolation of the Hemorrhage-Producing Fraction from *Serratia marcescens (Bacillus prodigiosus)* Culture Filtrate[1]

By M. J. SHEAR, *principal biochemist*, and FLOYD C. TURNER, *senior surgeon*, with the technical assistance of ADRIEN PERRAULT and THERESA SHOVELTON, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Certain bacterial products, when administered parenterally to animals bearing sarcomas, produce severe hemorrhage in the tumors within a few hours; subsequently the affected areas of the tumor become necrotic. The earlier literature on the effect of administration of bacterial preparations on tumors in experimental animals and in patients was summarized in a previous paper (*2, pp. 82–85*). A later and more extensive treatment of this subject was given in chapter VIII of Shwartzman's monograph on the phenomenon of local tissue reactivity (*3*).

Although relevant publications began to appear in 1882, interest in the effect of bacterial preparations on tumors lapsed after a few decades. It was revived in 1931 by the findings of Gratia and Linz (*4*), who injected *Bacillus coli*[2] culture filtrate into guinea pigs bearing a transplanted liposarcoma. The next year Shwartzman and Michailovsky (*5*) reported that parenteral administration of agar-washings filtrate of a meningococcus culture produced hemorrhage and necrosis in mouse sarcoma 180, that some of the tumors receded completely, and that hemorrhagic lesions were not produced in the normal tissues. Their findings were confirmed and extended by Shear (*2*) with material furnished by Shwartzman. In agreement with the latter's observation, it was found that meningococcus filtrate was highly toxic in the large doses required for destruction of enough tumor tissue to result in complete regression. Although smaller doses were tolerated better, they were relatively ineffective in producing complete regression of the tumors.

The separation of the hemorrhage-producing agent from toxic and inert contaminants was therefore attempted by Shear and Andervont (*6*), who obtained a highly potent concentrate from filtrates of broth cultures of *Escherichia coli* (*Bacillus coli*). Further purification (*7*) gave a preparation which produced hemorrhage in mouse sarcoma 37 in doses as low as 0.4 microgram.

The literature contains little information on the chemical nature of the agent responsible for the production of hemorrhage in mouse tumors. Somewhat more attention has been devoted to the purification and nature of the agent in bacterial preparations which produces the Shwartzman phenomenon. From agar-washings of cultures of *Bacillus typhosus*, Apitz (*8*)

[1] *A preliminary report* (1) *of this work was presented at the 1940 meeting of the American Society of Biological Chemists in New Orleans.*

[2] *In referring to organisms used in our own work we have employed Bergey's classification. As regards organisms employed by other workers, we have retained the designations used in the original publications in order to avoid confusion.*

obtained "Shwartzman-active" preparations of a nucleoprotein fraction and of an alcohol-precipitable, biuret-negative, Molisch-positive fraction, but they were found to be so unstable as to lead to the conclusion that "further attempts at their final purification seem useless." The chemical properties of Shwartzman-active fractionated materials were reviewed, as far as then known, by Shwartzman (*3, ch. III*). He concluded that the various efforts at concentration and purification suggested that they were either protein in nature or perhaps associated with proteins.

Andervont and Shear (*9*) produced the Shwartzman reaction in normal rabbits with 0.5 microgram of a biuret-negative, Molisch-positive concentrate, from filtrates of *Escherichia coli* (*Bacillus coli*) cultures, which had produced hemorrhage in mouse sarcomas in doses as low as 0.3 microgram. Glick and Antopol (*10*) produced the Shwartzman effect with a protein-free Shiga preparation, containing fatty and polypeptide materials in addition to polysaccharide, which was obtained from Morgan (*11*), and with protein-free carbohydrate fractions of *B. proteus*.

It is not yet known whether the same substance is capable of eliciting both hemorrhage in sarcomas and the Shwartzman phenomenon in rabbit skin, or whether preparations which produce both effects (*9*) contain two active agents, each of which is responsible for one of these phenomena. Consequently, further consideration here of the chemical fractionation of such materials will be restricted to those contributions in which the potency of the fractions was determined by their effect on tumors.

An alcohol-insoluble fraction which produced hemorrhage in mouse sarcomas was obtained from *Bacillus proteus*, *B. typhosus*, *B. coli*, *B. pyocyaneus*, and *B. prodigiosus* by Fogg (*12*) in an extension to the organisms themselves of techniques previously carried out on filtrates of bacterial cultures by others (*6*) in the same laboratories. Gardner, Bailey, and Hyde (*13*) injected into rats, bearing implants of Walker tumor 256, carbohydrate-containing fractions from 26 different bacteria, as well as a variety of other carbohydrate materials including one derived from yeast; hemorrhage in the tumors was produced with about half of the preparations tested. Since these materials were of complex nature, an active carbohydrate preparation from *Salmonella enteriditis* was hydrolyzed. Gardner, Bailey, and Hyde obtained a polysaccharide component which gave a faintly positive biuret test, and a "toxic component" which was insoluble in acetic acid and soluble in aklali and which contained little precipitable material. Hydrolysis was accomplished by heating the complex at 100° C. for 4 hours in 0.2N acetic acid. Under these conditions, heat alone without acid destroyed 95 percent of the hemorrhage-producing potency of the complex. Hemorrhage was produced with these hydrolytic products only when very large doses were employed. The authors, nevertheless, concluded that the hemorrhagic reactions were due to the polysaccharide-free component of the complex.

Walker and Handman (*14*) fractionated the saline washings of nutrient agar cultures of an organism stated to be probably *Salmonella typhi murium*. By repeated treatment with acetic acid, filtration, and precipitation with alcohol, a carbohydrate fraction was obtained which gave a negative biuret reaction. The bacteria themselves were extracted with 0.05N sodium hydroxide, and the alkaline extract was treated with acetic acid. The precipitate was repeatedly dissolved and reprecipitated, and washed with water, alcohol,

and ether. Hydrolysis of a portion of this nucleoprotein fraction gave nucleic acid.

These and other fractions which had "not been adequately identified or purified" were administered to tumor-bearing mice and rats and to normal rabbits. The fractions were all toxic, particularly the polysaccharide preparation. The tumors employed were sarcoma 180, carcinoma 63, several strains of transplantable carcinoma which had developed in their own mice, and the Walker rat tumor 256. The animals used included hybrid mice and rats, the dba and Swiss strains of mice, and a Slonaker strain of rats. Most of the fractions produced hemorrhage in the tumors, with complete regression in some instances; both sarcomas and carcinomas were affected similarly but, in agreement with the findings of others, the sarcomas were more sensitive. Of the more highly purified materials, the nucleic acid fraction was negative while the polysaccharide and nucleoprotein fractions were both potent. Walker and Handman stated (*14, p. 521*):

> The distribution of the tumor-affecting property among the nucleoproteins and polysaccharides might suggest that the active agent was some special substance associated with these fractions. If that be the case, it must be present in minute quantities, it must be exceedingly potent and inseparable from the bulk of the carrier by ordinary biochemical methods. The activity of both the polysaccharides and nucleoproteins explains the diverse results of previous attempts to separate the active substance.

Shapiro (*15*) studied the effect on tumor cells in vitro of the "toxic carbohydrate complex" from *Salmonella enteriditis* that was used by Gardner, Bailey, and Hyde (*13*). Tissue cultures of rat sarcoma 10 and Walker carcinoma 256 were employed. The explants were immersed in solutions containing the carbohydrate complex for periods ranging between 15 and 70 minutes and then transferred to the culture medium. The subsequent growth of these cultures was compared with the growth of control tumor tissue in the same medium without prior exposure to the bacterial substance. Under these conditions no effect on the growth of tumor tissue in vitro was noted following exposure to this agent. "Toxic carbohydrates" were also prepared by removing the organisms from liquid cultures by high speed centrifuging, precipitating the proteins in a 16-percent concentration of trichloracetic acid, and treating the supernatant solution with ethyl alcohol and sodium acetate. The preparations obtained in this way also were without effect on tumor cells in tissue culture although highly potent in the production of hemorrhage and necrosis in tumors in the intact animals.

The foregoing review of the literature shows that not much is known about the chemical nature of the bacterial products that produce hemorrhage in tumors. Attempts to isolate the active substances had yielded potent fractions, but further purification of these fractions had not been accomplished satisfactorily.

Two of the major difficulties in further progress in this direction have been the comparatively small amounts of active material available for chemical study, and the variability of the biologic response. It is relatively easy to discriminate between materials whose potencies differ by tenfold or a hundredfold. But when concentrates are further fractionated, the active agent is often distributed among all the fractions. In such cases the bioassay technique must be sufficiently sensitive to detect smaller differences in potency before it can be ascertained whether any given step in the fractionation is useful.

This paper records the results of work carried out between 1937 and 1940 on the preparation of large amounts of filtrates of cultures of *Serratia marcescens* (*Bacillus*

prodigiosus), and the isolation from them of highly potent concentrates. This organism was chosen for several reasons: it is employed in the preparation of "Erysipelas and prodigiosus toxins (Coley)"; it produces hemorrhage in mouse sarcomas; and it is nonpathogenic. Attempts were made to increase the hemorrhage-producing potency of the culture filtrates by various chemical and physical means. In some instances growth-promoting substances (indole-3-acetic acid, indole-3-propionic acid, nicotinic acid, or pantothenic acid) were added; in others chlorophyll or colchicine, which produces polyploidy and other effects in higher classes of plants, was added to the culture medium. The organisms were also grown in light of various intensities and wavelength distributions. In these exploratory experiments, however, no significant increase in the potency of the filtrates was detected by the bioassay method employed.

In the early stages of this investigation a nutrient broth medium was employed; later, a simple synthetic medium containing only inorganic salts and glucose was used. A bioassay technique was developed which provided roughly quantitative data on the potency of the fractions. In this way a fraction was obtained which gave a strongly positive Molisch test and a negative biuret reaction, and which produced hemorrhage in mouse sarcoma 37 in doses as low as 0.1 microgram. This material was obtained in quantities sufficient for further chemical and biologic investigation.

METHODS AND PROCEDURE

Preparation of Bacterial Filtrates

Organism

A specimen of chromogenic *Serratia marcescens* ("G. W.") was obtained on October 5, 1936, from the Division of Infectious Diseases, National Institute of Health. It was carmine-colored in the cultures grown on agar slants at room temperature, and colorless in cultures in fluid media at 38° C.

Stock cultures of the organism were maintained on agar slants at room temperature. The various batches of fluid media were inoculated, as needed, with organisms from these stock cultures.

Culture Medium

In the earlier stages of this work, a nutrient broth medium was employed as in the previous work with *Escherichia coli* (*Bacillus coli*) (*6*). When it was found that the numerous complex components in the medium itself complicated the separation of the active agent from inactive and toxic contaminants, simple synthetic media were employed. These were modifications of a medium employed by Gladstone (*16*) for the cultivation of *Bact. typhosum*. Magnesium sulfate and sodium bicarbonate were omitted from this medium, and the concentrations of some of the other constituents were changed somewhat. The composition of the medium used in this study was as follows:

Constituent:	Grams per liter
KH_2PO_4	5.0
NH_4Cl	.5
$(NH_4)_2SO_4$	.5
Glucose	2.5

The inorganic salts (reagent grade) were dissolved in distilled water, the pH was adjusted to neutrality with 1N sodium hydroxide, and the solution was allowed to stand. A small amount of insoluble material usually separated out, presumably the phosphates and sulfates of traces of heavy metals present as impurities in the salts. This material was filtered off, and the glucose was then added. After making the solution up to volume, it was passed through a Berkfeld filter in the early experiments. In later work the salt

solution was sterilized by autoclaving once, and the glucose solution was made sterile by fractional sterilization in the autoclave or by filtration through a Seitz filter.

Bacterial Filtrate

The medium was heavily seeded with the organism from 24-hour agar Blake bottle cultures and placed in the incubator room at 37° C. for about a week. The bulk of the organisms was removed by passing the culture through a Sharpless supercentrifuge. The remaining organisms were removed by Berkfeld filtration The filtrate obtained in this way served as the starting material.

Bioassay Technique

Sarcoma 37 was maintained, by serial transplantation, subcutaneously in dba mice. Tumor tissue from this source was inoculated just below the dermis (*17*) of ABC mice obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The cutaneous tumors protruded from the abdominal surface and presented a large area of surface for observation of induced hemorrhage. Purina dog chow and tap water were freely available to the animals at all times.

The mice were employed in the assays 6 to 8 days after implantation of tumor tissue. When the tumors were too young they did not respond to the action of the bacterial agent, as had been found by earlier workers. When the tumors were too old or too large, localized areas of brown discoloration developed on the outer surface and obscured the hemorrhage unless it was pronounced. This discoloration occasionally resembled the necrosis which developed after a mild degree of induced hemorrhage. Tumors exhibiting this discoloration were therefore not employed in the bioassays, although they had some use in exploratory experiments in which large doses were given and in which severe hemorrhage was produced.

The volume administered was brought to 0.5 cc. with a 0.9-percent solution of sodium chloride, and the solutions were injected intraperitoneally. Each fraction or preparation was usually tested at three dose levels differing threefold from each other, e. g., 0.3 cc. of 1:1,000 dilution, and 0.1 and 0.3 cc. of 1:100 dilution. About five tumor-bearing mice were usually employed for each dose. While this technique was not sufficiently delicate to detect small differences in potency, it was found useful in differentiating between fractions of low potency, which were discarded, and those of high potency, which were subjected to further fractionation.

RESULTS

Filtrates From Nutrient Broth Medium

The method previously employed (*6*) for the separation of the hemorrhage-producing agent from nutrient broth cultures of *Escherichia coli* (*Bacillus coli*) was applied to analogous broth cultures of *Serratia marcescens*. The addition of 2.5 gm. of KH_2PO_4 per liter of filtrate, followed by addition of lime water (*18*) to approximately pH 9.5, yielded a flocculent precipitate which brought down about two-thirds of the active material. Repeated treatment of the supernatant solution yielded additional amounts of active material in the subsequent basic calcium phosphate precipitates. The final supernatant solution was discarded.

The precipitates were combined and dissolved in 2.5N hydrochloric acid, and the small amount of insoluble matter was removed by filtration. Reprecipitation by the addition of 2.5N sodium hydroxide left the supernatant solution inactive, while the potent material was contained in

the precipitate. The active material was separated from the large amount of calcium and phosphate by addition of six volumes of 95 percent ethyl alcohol to the acid solution (pH 2) of the precipitate. The treatment was repeated until the precipitate gave a negative test for calcium.

This procedure yielded an active fraction contained in a relatively small volume; but while it removed much of the impurities originally present, the treatment of large volumes of filtrate by repeated addition of large volumes of lime water was cumbersome. Various modifications of this technique were tried on batches of filtrate totaling over 300 l. in an attempt to increase the efficiency of the concentration, but a satisfactory procedure was not obtained. Furthermore, the active fractions after alcohol precipitation were brown, instead of white as was the case when this procedure was employed with filtrates of *Escherichia coli* (*Bacillus coli*) cultures (*7*).

Filtrates From Simple Synthetic Medium

Much of the difficulty in the purification resulted from the large amount of complex material originally present in the nutrient broth medium. An entirely different medium was therefore sought for the culture of the organism. Following the method of Morell and Shwartzman (*19*), it was found that *Serratia marcescens* would grow and would elaborate the hemorrhage-producing agent in the dialyzate of a nutrient bouillon culture medium. This demonstrated that *S. marcescens* did not require protein or other high-molecular components for growth and for production of the active agent.

Since other workers had found that various organisms could be grown in simple synthetic media, modifications of the one used by Gladstone (*16*) for cultivation of *Bact. typhosum* were tried. Magnesium sulfate was omitted from his formula, inasmuch as we had obtained indications that magnesium decreased the potency of the hemorrhage-producing agent. Sodium bicarbonate was omitted for reasons of simplicity, since loss of CO_2 would change the pH and since maintenance of constant composition of the air above the cultures would be cumbersome in large-scale production of filtrate.

By employing the medium described in a foregoing section, potent filtrates were obtained in a solution containing only inorganic salts and glucose. The activity of these filtrates was approximately the same as that of filtrates obtained from nutrient broth cultures. The end point in the titrations with tumor-bearing mice was usually about 0.4 cc. of 1:100 dilution of the filtrate.

With large doses of active material, severe hemorrhage was produced in the tumors of all mice. As the dose was decreased, the severity of the hemorrhage was less and the area of involvement of tumor tissue by hemorrhage decreased; moreover, the number of mice showing unaffected tumors increased, until with sufficiently small doses the tumors were negative for hemorrhage in all the mice. The end point was defined as that dose which produced grossly perceptible hemorrhage in the tumors of half of the mice. Table 1 gives a sample assay in which the end point was estimated to be about 0.25 cc. of 1:100 dilution of the original filtrate. A "mouse tumor unit" (m. t. u.) was defined as the amount of activity contained in the end point dose. Hence this particular preparation contained 400 m. t. u. per cubic centimeter or 400,000 m. t. u. per liter. For calculations of total amounts of activity in large volumes of material, e. g., hundreds of liters, it has been found convenient to employ a larger

TABLE 1.—*Sample assay of filtrate, from cultures of* Serratia marcescens *in simple synthetic medium, for potency in producing hemorrhage in mouse sarcoma 37*

Dose of original filtrate		Mice injected	Mice with induced hemorrhage [1] in tumor				
Dilution	Volume		++++	+++	++	+	−
	Cubic centimeters	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
1:10	0.3	5	1	3	1		
1:10	.1	5		1	2	2	
1:100	.3	5			1	2	2
1:100	.1	5			1		4
1:1,000	.3	5					5

[1] The severity of induced hemorrhage is indicated by the number of plus signs; the minus sign denotes a negative response. The end point was estimated to be approximately 0.25 cc. of 1:100 dilution. This filtrate, therefore, contained 400,000 m. t. u. (or 0.4 M. T. U.) per liter.

unit (M. T. U.) in which 1 M. T. U. = 1,000,000 m. t. u.

CONCENTRATION OF THE ACTIVE AGENT

The results described henceforth deal with data obtained on material from cultures grown in the simple synthetic medium. Filtrates obtained in this way were clear, almost colorless, watery solutions. An attempt was next made to concentrate the active agent by a more convenient method than precipitation along with basic calcium phosphate, in view of the unwieldy volumes obtained on repeated addition of lime water to large amounts of filtrate.

Precipitation By Chloroform

To remove the protein present, the method of Sevag and coworkers (*20*) was tried inasmuch as they stated that this technique results in the formation of a water-insoluble, chloroform-protein gel. Repeated treatment of the filtrate with excess chloroform and a small amount of *n*-butyl alcohol gave successively smaller amounts of white emulsion between the aqueous and chloroform layers. It was expected that the active material would reside in the aqueous layer, since previous work with *Escherichia coli* had shown that the purified material was carbohydrate in nature and gave a negative biuret reaction for protein.

Bioassay, however, showed that the aqueous layer contained only a small fraction of the activity, and that most of the potent material was concentrated in the chloroform-protein emulsion. Before testing the two solutions, the chloroform and butyl alcohol were removed from both fractions at 38° C. In one experiment of this nature, the end points obtained were 0.1 cc. of 1:100 dilution for the aqueous solution of the precipitate, and 0.5 cc. of 1:10 dilution for the aqueous supernatant solution. Table 2 gives the total volumes of the solutions, and the potencies in mouse tumor units, as compared with the original filtrate. About 10 percent of the activity remained in the supernatant solution, whereas the remainder had been concentrated in the emulsion layer by the treatment with chloroform. Although the total activity of the two fractions appears to be greater than that of the initial material, the difference is not significant since it is within the limit of sensitivity of the bioassay method.

In this instance, the small volume of filtrate (500 cc.) was treated with chloroform in a shaking machine. In view of the promising results, which were confirmed by numerous repetitions, large volumes of filtrate were treated with

TABLE 2.—*Distribution of potency in production of hemorrhage following precipitation with chloroform*

Material	Volume	End point (approximate)		Total potency (approximate) in m. t. u.
		Dose	Dilution	
	Cubic centimeters	*Cubic centimeters*		
Original filtrate	500	0.5	1:100	100,000
Supernatant solution	480	.5	1:10	9,600
Precipitate [1]	111	.1	1:100	111,000

[1] Dissolved in water.

chloroform. Precipitation was effected by vigorous mechanical stirring at room temperature, which was found more convenient and more efficient for the large volumes handled.

Treatment of a 6.6-l. batch of filtrate for example, yielded similar results; more than 80 percent of the potent material was precipitated by treatment with chloroform. After removal of this reagent from the emulsion layer, the precipitate was contained in 1 l. of aqueous solution. The active fraction was reprecipitated from this solution with chloroform and again obtained in aqueous solution, this time in a still smaller vloume.

Other Precipitants

Aliquots of an aqueous solution of material which had been twice precipitated with chloroform were treated with various reagents. Sodium tungstate and sulfuric acid gave a small amount of dark brown precipitate which contained only a small fraction of the potency; the activity remained in the supernatant solution for the most part. Trichloracetic acid gave no precipitate. On addition of ethyl alcohol to the aqueous solution of the chloroform precipitate, a flocculent precipitate of the active material was obtained.

Treatment of a highly potent concentrate with two volumes of methyl alcohol yielded a precipitate containing an appreciable fraction of the total potency. The bulk of the activity remained in the supernatant solution from which it could be precipitated by acidification with HCl; this precipitate was soluble in aqueous NaOH. However, further attempts at fractional precipitation with methyl alcohol and HCl resulted in complete destruction of potency. The material became insoluble in aqueous NaOH and lost all its activity.

Frothing

Previous workers had employed butyl or amyl alcohol in the chloroform precipitation to minimize frothing. Since the protein content of the filtrates from cultures grown in the simple synthetic medium was so low, it was expected that addition of a surface-tension-reducing substance might not be necessary. Precipitation with chloroform alone was therefore tried and was found to give satisfactory results inasmuch as little frothing occurred.

The ratio of chloroform to filtrate was varied in a number of experiments in which no anti-froth compound was added. It was found that a ratio of about 1 volume chloroform to 4 volumes of filtrate gave good results. In the subsequent manipulations about five volumes of ethanol were used, together with one volume of ethyl ether and some salt to make precipitation more complete. At first sodium acetate was employed; in later preparations sodium chloride was used. The alcohol precipitate was dissolved in a small volume of water and reprecipitated by alcohol in the same way. The final precipitate was usually dissolved in water to give an approximately 1-percent solution.

One preparation (B–10) had an end point of 0.5 cc. of 1:1,000, i. e., it contained 2,000 m. t. u. per cubic centimeter which was about 10 times the potency of the original filtrate. This preparation contained 15 mg. protein per 100 cc. of a 1-percent solution of the active fraction; in other words, protein constituted 1.5 percent of the total dry weight of this fraction.

Dialysis

A concentrate with an end point of 0.3 cc. of 1:1,000 (containing about 3,300 m. t. u. per cubic centimeter) was dialyzed

through cellophane against water for 5 days over a wide pH range. Five aliquots were brought to the following pH values before dialysis: 1, 4, 5, 7, 9, and 10. On completion of the dialysis, the dialyzates and the nondialyzable fractions were neutralized and assayed for potency.

In all instances the dialyzates were devoid of potency. The activity of the dialyzed material was unimpaired except for the aliquot dialyzed at pH 1. It may be noted, however, that in a subsequent experiment (*21*) dialysis at pH 1 did not cause a decrease in potency.

Concentrates of High Potency

One batch of filtrate (D) was repeatedly precipitated with chloroform and with ethyl alcohol. The precipitates were obtained in successively smaller volumes until the final product was contained in 91 cc. The data are given in table 3.

Evaporation of this preparation to dryness yielded 26 mg. total solids (active material and salt) per cubic centimeter. One m. t. u. therefore contained 0.26 microgram, which compared favorably with the value of 0.4 microgram per m. t. u. obtained previously with a concentrate from cultures of *Escherichia coli* (*7*).

With another batch of filtrate (G) a concentrate was obtained with an end point of 0.7 cc. of 1:10,000 dilution. Since the final volume was 100 cc., this concentrate represented only about a 20-percent recovery of the total potency present in the original filtrate (table 3).

Batch K (table 3) was treated with chloroform, and the precipitate dissolved in water and reprecipitated with the same reagent. It was then precipitated twice with three to five volumes of ethyl alcohol; flocculation and sedimentation were promoted by the addition of ether and of dry sodium chloride. The final precipitate was taken up in 360 cc. of water. Since this concentrate contained 10 mg. of total solids per cubic centimeter, the minimum hemorrhage-producing dose contained 0.1 microgram of the active fraction.

To facilitate separation from various types of contaminants, one alcohol precipitation was carried out at about pH 9.5 and a second at pH 2.

Variations of the same basic procedure were employed with two other batches (L and M) of filtrate with volumes of somewhat more than 100 l. each. The end points of the concentrates obtained were close to those previously obtained, and the total yield of potency was considered satisfactory. Table 3 summarizes the results obtained with these batches. On the whole, about half of the initial potency present in the 548 l. of filtrate was obtained in the 1.168 l. of final solution. The potency of the concentrated material was, on the average, about 250 times that of the original filtrate.

The most potent solution obtained in the course of this work had an end point of 0.75 cc. of 1:100,000 dilution. This value was obtained consistently in three bioassays performed on different occasions. The potency of this concentrate was therefore about 130,000 m. t. u. per cubic centimeter.

Table 3.—*Potency of concentrates obtained for various batches of* Serratia marcescens *filtrate*

Batch	Filtrate used				Concentrate obtained				
	Volume	End point		Total potency in M. T. U.	Volume	End point		Total potency in M. T. U.	Potency recovered
		Volume	Dilution			Volume	Dilution		
	Liters	*Cubic centimeters*			*Liters*	*Cubic centimeters*			*Percent*[1]
D	36	0.3	1:100	12	0.091	0.1	1:10,000	9	75
G	36	.5	1:100	7	.100	.7	1:10,000	1.5	20
K	264	.4	1:100	66	.360	.1	1:10,000	36	55
L	108	.5	1:100	22	.227	.2	1:10,000	11	50
M	104	.5	1:100	22	.390	.2	1:10,000	19	85
Total	548				1.168				

[1] Approximate.

Chemical Properties

The concentrates were usually prepared so as to contain about 10 mg. of non-dialyzable material per cubic centimeter since a 1-percent solution appeared to be near the upper limit of solubility of the active material. The potent fractions prepared according to the procedure outlined above gave a strongly positive Molisch test for carbohydrate, and the best preparations gave negative results in the biuret, trichloracetic acid, and sulfosalicylic acid tests for protein.

Analysis [3] of concentrate K–8d gave the results shown in the following tabulation:

Constituent:	Content *Percent*
Carbon	47.55, 47.69
Hydrogen	7.30, 7.26
Nitrogen	3.85, 3.84
Acetyl	7.22, 6.95
Ash	3.82

These analytical values are similar to those obtained for other bacterial polysaccharides (*11*, *22*), and for the blood group A specific substance from commercial peptone (*23*). The end point of this preparation, as determined in repeated bioassays, was 0.1 cc. of a 1:10,000 dilution of a 1-percent solution. One m. t. u. therefore contained 0.1 microgram of the active fraction.

Evaporation at atmospheric pressure or in vacuo gave a glassy residue when the solutions were dried at room temperature. Drying in this way resulted in loss of ready solubility in water and in destruction of potency. The dried glassy material was soluble in 2.5N NaOH and somewhat soluble in pyridine. The lyophylized material was readily soluble in glycerol (*11*). Lyophilization, first carried out by Dr. Julius White, gave a fluffy, amorphous residue with solubility in water unchanged and with the potency unimpaired.

The value for total solids was usually obtained by drying in a desiccator over $CaCl_2$ at room temperature. For example, one dialyzed concentrate (K–8d) yielded a value of 10.3 mg. per cubic centimeter when dried in this way. Precipitation with acetone followed by drying at 50° in the oven gave a value of 9.6 mg. per cubic centimeter. Lyophilization of another aliquot of the same preparation yielded 12.0 mg. per cubic centimeter. Further drying of the lyophilized material in a desiccator removed additional water with concomitant destruction of potency.

One of the properties previously noted in the case of the polysaccharide fraction obtained from *Escherichia coli* filtrates (*7*) was the formation of a permanent foam. This characteristic was observed in the early stages of the manipulations and was retained by the purest materials obtained in this study.

The active agent was stable. It retained its potency, both in dilute or concentrated aqueous solution and in the dry state (lyophilized), for long periods when stored in the refrigerator. In some cases, concentrates were kept for 2 years without destruction of potency. The activity was not lost in manipulations involving pH changes between 3 and 10. Elevated temperatures produced a loss of potency. Freezing the solutions in a refrigerator sometimes resulted in destruction of potency, but at other times the preparations were still potent after being frozen.

Toluene was usually employed as preservative, since the solutions otherwise often became contaminated, particularly with fungi. However, several preparations, contaminated with unidentified

[3] *The microanalytical determinations were made by the Arlington Laboratories, Chagrin Falls, Ohio.*

fungi, were found on test to have suffered no impairment of potency.

Treatment with activated charcoal (norite) to remove colored impurities effected an improvement in the color but in addition resulted in removal of most of the potency both from purified concentrates and from the original filtrates. Aluminum hydroxide precipitation also removed the activity from solution.

Toxicity

In the early stages of the fractionation, materials were obtained that were both potent and highly toxic. Fractionation of such materials usually yielded fractions which were highly potent and only slightly toxic, and others which were less potent and highly toxic. No systematic study was made of toxicity, but the impression was gained that the agent which produced the hemorrhage in tumors had a relatively low intrinsic toxicity. This was borne out in subsequent experiments (*24*).

In a few experiments, doses of active concentrates large enough to produce death were given. They were found to range from about 100 to 1,000 times the minimum hemorrhage-producing dose.

Miscellaneous Bacterial Preparations

A number of miscellaneous bacterial materials were tested from time to time as they became available to us. These had been prepared for various special purposes by a variety of techniques, hence the negative results obtained with some of these preparations should not be construed as evidence that fractions obtained from these organisms by other methods would also be ineffective in producing hemorrhage in tumors.

Hemorrhage was produced in sarcoma 37 in ABC mice with three preparations of paratyphoid vaccine obtained from Dr. E. S. Robinson, of the Antitoxin and Vaccine Laboratory, Massachusetts Department of Public Health. All the other materials tested gave negative results for hemorrhage. The preparations and their sources were: purified tuberculo-protein (Sharpe and Dohme); carbohydrates from type I, type II, and avian pneumococcus (Dr. M. G. Sevag); *Bacillus proteus* carbohydrate (Dr. M. G. Sevag); *B. influenza* carbohydrate (Dr. H. Mueller); streptococcal toxins (Dr. M. V. Veldee, Dr. Alice Evans, Dr. A. Stock, and Dr. Maud L. Menten). A toxin prepared from *Streptococcus viridans* (strain 603 of the National Institute of Health) also failed to produce hemorrhage in the tumors.

Hemorrhage in sarcoma 37 was produced with a commercial preparation of "Coley's mixed toxins" of *Erysipelas streptococcus* and *Bacillus prodigiosus*. The potency was low, however, for this material was found to contain only 100 m. t. u. per cubic centimeter. The filtrates used as the starting material in the present experiments had an average potency of about 250 m. t. u. per cubic centimeter while the concentrates listed in table 3 had an average potency of about 50,000 m. t. u. per cubic centimeter. The most potent preparation contained about 130,000 m. t. u. per cubic centimeter.

DISCUSSION

The experiments recorded here were carried out in mice bearing transplants of sarcoma 37. While it is generally agreed that sarcomas respond more readily to the action of bacterial substances than do carcinomas, it is by no means correct to maintain that carcinomas are not affected by these agents. Thus, in 1933 Apitz (*25*) purposely chose to work with a carcinoma (Ehrlich) because previous workers had employed only sarcomas, and found that this mouse carcinoma exhibited hemorrhage and necrosis following administra-

tion of bacterial filtrate. In 1935 Duran-Reynals (*26*) reported that bacterial toxins produced local reactions in spontaneous mouse carcinomas. In some cases there was regression of the tumor followed by recurrence; in others there was permanent regression. Duran-Reynals thus extended to spontaneous carcinomas the observations he had reported 2 years earlier on transplanted carcinomas and sarcomas of mice and rats. Jacobi (*27*) reported that he had obtained a hemorrhagic and necrotic reaction in the tumors of 20 animals bearing the Flexner-Jobling rat carcinoma, following the injection of a diluted *Bacillus typhosus* filtrate. More recently Nagasima (*28*) described the production of hemorrhage in 3 transplantable rat tumors following injection of *B. coli* filtrate; positive results were obtained not only with Fujiwara's rat sarcoma but also with Sasaki's rat hepatoma and with Bashford's carcinoma.

Little is known about the mechanism of the action of these bacterial agents on tumors. Attention has been directed mostly to the vascular system, but Apitz concluded that there was a primary edema of the tumor cells. Some of Nagasima's experiments were stated to reveal changes in the tumor cells which preceded those in the blood vessels. Andervont and Shimkin (*29*) reported that ascorbic acid prevents the appearance of hemorrhage in, and resultant regression of, transplanted tumors treated with *Serratia marcescens* (*Bacillus prodigiosus*) filtrate; a less pronounced inhibition was obtained with cysteine. They postulated that the bacterial filtrate "by lowering suddenly the ascorbic acid content of the tumor, weakens its fragile capillaries, with resultant hemorrhagic extravasation." However, in more recent experiments (*21*), no inhibition of hemorrhage was obtained with ascorbic acid, cysteine, or glutathione when the active polysaccharide fraction from *Serratia marcescens* filtrate was employed as the hemorrhage-producing agent.

Because of its vasodilator effect histamine, both alone and with bacterial preparations, was also tested for its action on the production of hemorrhage in tumors. Apitz (25) gave sublethal doses of histamine to mice bearing implants of the Ehrlich carcinoma but did not obtain hemorrhage in the tumors. Shear (*2*) did not obtain hemorrhage in, or regression of, sarcoma 37 in mice with repeated administration of large doses of histamine. Andervont and Shear [4] administered histamine prior to, with, and subsequent to the injection of *Escherichia coli* (*Bacillus coli*) filtrate; no effect on the hemorrhage-producing action of the filtrate was observed. On the other hand Turner [5] found that small amounts of histamine hydrochloride inhibited hemorrhage production in sarcoma 37 when administered together with or shortly before the injection of *Serratia marcescens* filtrate; it was without effect when injected after the administration of the filtrate. This inhibition was not obtained with the free base. Barrett (*30*) reported that histamine hydrochloride by itself produced hemorrhage and necrosis in tumors of mice bearing sarcoma 37. With this same technique, however, Turner did not obtain hemorrhage.

Peptone, which also produces protracted shock, gave negative results in the hands of Apitz (*25*) and of Shear (*2*); Barrett (*30*), however, stated that doses large enough to be toxic produced extensive reactions in the tumors of some of the mice.

Anaphylactic shock has been considered as one of the possible mechanisms in the production of hemorrhage in tumors by

[4] *Andervont, H. B., and Shear, M. J.: Unpublished work (1936).*

[5] *Turner, F. C.: Unpublishe work (1939).*

bacterial substances. Apitz (*25*) obtained negative results in exploratory experiments in which attempts were made to induce hemorrhage in Ehrlich mouse carcinoma by producing anaphylactic shock with horse serum, although this tumor gave the hemorrhagic response to bacterial filtrate. Shwartzman (*3*) has discussed in detail the relationship between hemorrhage production in tumors and the Shwartzman phenomenon. Gardner, Bailey, and Hyde concluded (*13, p. 14*): "The local and systemic reactions of tumor bearing rats injected with the soluble specific substances of certain microorganisms would appear to find an explanation in terms of hypersensitization and generalized anaphylaxis." Barrett (*30*) found that mice previously sensitized to horse serum showed signs of shock, and hemorrhage and necrosis in the tumors, following a provocative injection of horse serum given 14 days after the sensitizing dose. In this connection, it may be of interest to recall that Shear (*2*) had obtained hemorrhage in, and destruction of, mouse tumors that had received horse serum injected in such a way that sensitivity presumably did not develop.

Apitz (*25*) used a variety of agents which produced hemorrhage in normal tissues, but in no case did these agents produce hemorrhage in the tumors. On the other hand, filtrates of bacterial cultures, agar, antigen-antibody mixtures, and antihomologous serum (i. e., Shwartzman active agents) were capable of inducing hemorrhage in tumors. Highly suggestive results were obtained by Antopol with fractions obtained from mammalian tissues. Extracts of sterile human tumors were tested by Antopol (*31*) in the skin of rabbits; he found that preparatory and provocative factors for the Shwartzman reaction could be demonstrated in tumor tissue. Antopol and Glick (*32*) next found that bovine pancreas and testis and rabbit liver could exhibit both preparatory and provocative potency in the Shwartzman phenomenon. Dr. Antopol kindly provided us with four of these extracts which had given positive results in the Shwartzman reaction. Dilute solutions of these extracts were injected into mice bearing sarcoma 37 (*21*); the doses employed contained 0.2 and 0.02 mg., respectively, of the dry extracts. At these levels, essentially negative results were obtained with three extracts. The extract from bovine pancreas, however, induced hemorrhage in the tumors in a single dose of 0.2 mg. The possibility that certain mammalian tissues may contain substances, not derived from contaminating microorganisms, which are capable of producing hemorrhage in tumors is of sufficient interest to warrant further study.

Lewisohn (*33*) reported that extracts of bovine pancreas, testis, liver, and heart did not produce hemorrhage in mouse sarcoma 180, but that extracts of the spleen and of the kidney did. The kidney extract also elicited the Shwartzman phenomenon, while the spleen extract did not. Complete regression of the tumor was obtained in 60 percent of 281 treated animals as compared with 8 percent of spontaneous regressions in 290 control mice.

In subsequent work with extracts of "healed spleen" of the mouse, Lewisohn, Leuchtenberger, and Laszlo (*34*) did not encounter the hemorrhage previously obtained with bovine spleen extract. Nevertheless, complete regression was obtained in 39 of 57 mice bearing sarcoma 180, as compared with regressions in 8 of 87 control tumor-bearing mice. Complete regression of spontaneous mammary gland carcinoma was also obtained in a significant proportion of the mice treated with healed spleen extract. In subsequent papers from Lewisohn's laboratory dealing with regression of mammary gland tumors of the mouse following treatment with yeast ex-

tract, no mention of induced hemorrhage was found.

Colchicine is another substance which is capable of inducing hemorrhage in tumors. This finding of Boyland and Boyland (*35*) was confirmed by Brues, Marble, and Jackson (*36*) and by Andervont (*37*). Colchicine, however, elicits hemorrhage in tumors only in doses close to the lethal, whereas the bacterial substances are effective in amounts much smaller than the lethal dose. Andervont found that these two agents supplemented each other in eliciting hemorrhage in grafted tumors; however, their combined action was no more effective than either agent alone in producing regression of the tumors.

The remarkably selective effect produced in tumor tissue by certain bacterial preparations gave rise to a number of interesting questions. The chemical nature of the active agent, the mechanisms involved in the production of the hemorrhage and the necrosis, and the reasons for the high sensitivity of certain types of tumors to such agents and for the resistance of other types of tumors, are but a few of these questions. Although hemorrhage and necrosis were readily and reproducibly obtained, few tumors regressed completely except under specialized conditions. The possibility remained, however, that purified preparations freed from toxic and inactive contaminants might prove useful in further attempts at therapy. Of interest in this connection are the reports of cases in which malignant tumors were alleged to have regressed completely after patients spontaneously contracted certain infectious diseases, particularly erysipelas, and of the allegedly favorable results claimed, in a small proportion of cases, by William B. Coley and by others who employed Coley's mixed toxins.

When interest in the effect of bacterial preparations on experimental tumors was revived about a decade ago, it was soon found that small doses were ineffective in producing complete regression and that large doses were too toxic. Several attempts were then made to surmount these difficulties. Thus, Shwartzman (*38*) injected *Bacillus typhosus* filtrate, and mixtures of this filtrate with antityphoid horse serum, into mice bearing sarcoma 180. A high proportion of the mice died within 24 hours after receiving the filtrate alone, whereas protection against early death was afforded when the injection was given with homologous antiserum; at the same time the hemorrhage and necrosis in the tumor tissue remained undiminished. Shwartzman, Morell, and Sobotka (*39*) employed dialysis to remove impurities of low molecular weight present in bacterial filtrates. Their fractionation procedure effected about a threefold increase in reacting potency (in the Shwartzman phenomenon in rabbit skin) per milligram of dry weight and of nitrogen, but attempts to purify the concentrates by fractional precipitation with dilute alkali, acid, and alcohol were unsuccessful. Morell and Shwartzman (*19*) then employed dialyzates of nutrient broth as culture media. A plain veal infusion broth, containing 1-percent peptone, was dialyzed through cellophane; the organisms were then cultivated in the dialyzate. These cultures were purified by dialysis through cellophane against an 0.85-percent solution of sodium chloride. The active principle always remained nondialyzable.

In the present investigation large amounts of relatively pure starting materials were obtained by employing a simple synthetic culture medium. Concentration was effected by vigorous stirring of the filtrate after addition of chloroform; this precipitated the potent material in the form of an emulsion which collected between the aqueous and the

chloroform layers. The emulsion yielded a concentrated aqueous solution of the active agent after removal of the chloroform. Precipitation with ethyl alcohol, followed by dialysis, gave a fraction which produced hemorrhage in mouse sarcomas in doses as low as 0.1 microgram. These fractions were rich in polysaccharide; moreover, some specimens gave negative reactions in tests for proteins.

In a recent paper Zahl and coworkers (*40*) were led to the conclusion that the "tumor-hemorrhagic agents produced by many gram-negative bacteria are probably identical with a polypepetide component of the complete endotoxin antigen." This opinion, however, was reached on the basis of analogy. No new chemical data were presented in support of this hypothesis.

According to Gerber and Bernheim (*41*), who studied the morphologic changes in mouse sarcoma 180 after injection of meningococcus filtrate, the effect appeared to be directly on the tumor cells. These authors stated:

> Although the hemorrhage is diffuse, involving almost the entire tumor, it is neither massive nor apparently severe enough to cause the widespread necrosis which is seen within twenty-four hours after injection of the filtrate. * * * The inflammatory reaction seen about the tumor in controls appears to be intensified in treated animals, yet on the whole it does not seem to be of sufficient intensity to account for the death of the tumor.

In agreement with the findings of others, they also stated:

> The effect of the filtrate on the tumor appears to be essentially one of direct action on the cells.

The selective effect on tumors of certain substances of bacterial origin make it of interest to obtain further information regarding their chemical composition and the nature of the mechanisms involved their action. The experiments described here provided a method for obtaining a stable, relatively pure, active fraction of high potency in amounts which made attempts at further purification feasible.

SUMMARY

A simple synthetic medium containing only three inorganic salts and glucose was found to be adequate for the cultivation of *Serratia marcescens* (*Bacillus prodigiosus*) with accompanying production of the substance which produces hemorrhage and necrosis in mouse tumors.

After removal of the organisms, the active agent in the filtrate was readily concentrated in a small volume by precipitation with chloroform. Further purification was facilitated by the absence of the numerous complex contaminants ordinarily contributed by the usual nutrient broth medium itself. Repeated precipitation with ethyl alcohol, followed by dialysis, gave highly potent preparations of the active agent.

These active fractions were rich in polysaccharide, and the best preparations gave negative results in tests for protein. Between pH 1 and 10, none of the active agent dialyzed through cellophane; none was destroyed except at the highest acid concentration. Lyophilization did not impair the potency.

The most active concentrate obtained in these experiments had a potency of 130,000 m. t. u. per cubic centimeter as contrasted with one of 100 units per cubic centimeter for a commercial preparation of Coley's mixed toxins. The minimum hemorrhage-producing dose contained 0.1 microgram of total solids.

The lethal dose of the various concentrates ranged from 100 to 1,000 times the minimum hemorrhage-producing dose.

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Chemical Treatment of Tumors. VI. Method Employed in Determining the Potency of Hemorrhage-Producing Bacterial Preparations

By M. J. SHEAR, *principal biochemist*, ADRIEN PERRAULT, *scientific aide*, and JOHN R. ADAMS, Jr., *assistant chemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

The method described in the previous paper (*1*) was found useful in discriminating between the activities of materials which differed widely in their potency in producing hemorrhage in mouse sarcomas. Difficulties were encountered, however, when the potencies were of the same order of magnitude. While at times the bioassays yielded good results, on other occasions discrepancies were noted, and repetition of determinations of potency sometimes gave widely discordant results. Often a small dose produced hemorrhage in a greater percentage of the tumors than did large doses of the same material in the same assay experiment. Sometimes the tumors of an entire batch of mice appeared to be refractory to hemorrhage production.

This paper reports a modified technique which yielded more satisfactory results. Testing of the tumor mash for bacterial sterility, increasing the number of mice used per dose level, examining the interior of the tumor for hemorrhage, and including a standard material for reference in each set of bioassays were the measures which were successful in reducing the discrepancies encountered previously.

MATERIALS AND METHODS

Dba and albino ABC mice, procured from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were used. Purina dog chow and tap water were freely available at all times.

The dba mice were employed to provide a stock supply of tumor material; sarcoma 37 was maintained subcutaneously in these mice. For the bioassays, healthy-looking tumor tissue was carefully dissected from the stock tumors and pressed through a mincing machine. The minced tissue was implanted just below the skin of albino ABC mice according to the method of Andervont (*2*). The mice were employed in the assays 6 to 8 days after inoculation of tumor mash.

Aqueous solutions of the materials assayed were injected intraperitoneally, in dilutions which varied threefold from one another. The volume of solution administered was between 0.1 and 0.5 cc. The tumors were examined for hemorrhage the day following the injection.

The tumors of untreated mice, and of those receiving active preparations in doses too low to induce hemorrhage, were pearly gray in color and usually were free of macroscopic hemorrhage. The active material produced effects which were proportional to the amount given. Border-line doses caused hemorrhage in a small area of the tumors in some mice, while other tumors remained negative. As the dose was increased, the percentage of hemorrhagic tumors increased, and in these

tumors the severity of the hemorrhage was greater and the area of tumor involved was larger. Minimum detectable hemorrhage is indicated in the tables by +. Hemorrhage induced in about a quarter of the tumor is indicated by + +, in more than half of the tumor by + + +, and in all or almost all of the tumor by + + + +. Tumors which were negative as regards hemorrhage are designated by a minus sign.

The end point was defined (*1*) as that dose which produced grossly perceptible hemorrhage in the tumors of half of the mice. With dose levels at which half of the tumors remained negative, the positive tumors usually exhibited only a mild degree of hemorrhage, i. e., + or ++. In estimating the end point all positive tumors were given the same weight; an occasional tumor showing ++++ hemorrhage, with a marginal dose, counted for no more than the other positive tumors which usually exhibited + or ++ hemorrhage. A mouse tumor unit (m. t. u.) was defined as the amount of activity contained in the end-point dose.

RESULTS

Number of Mice

On assaying materials whose potencies differed by tenfold or more, it was relatively easy to get useful results with five mice per dose level. But when a given active material was further separated into fractions having, for example, 10, 30, and 60 percent, respectively, of the potency, it was found necessary to employ more mice for each dose if satisfactory results were to be secured.

Table 1 gives illustrative assays, with marginal doses of various preparations, carried out in 3 instances (experiments 1 to 3) with 5 or 6 mice per group, and in 3 others (experiments 4 to 6) with 10 mice per group. Anomalous results were obtained more often when the smaller number of animals was employed. As was to be expected, the results were less variable when 10 mice were used per group. Even

TABLE 1.—*Sample assays of specimens of the hemorrhage-producing fraction from* Serratia marcescens *culture filtrates illustrating the effect of the number of mice employed upon the results obtained*

Experiment	Dose of concentrates [1]		Mice used	Mice with induced hemorrhage [2] in tumor				
	Dilution	Volume		++++	+++	++	+	−
		Cubic centimeter	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
1	1:10,000	0.1	6				2	4
	1:10,000	.3	6		4	1		1
	1:1,000	.1	5		2			3
2	1:10,000	.1	6				1	5
	1:10,000	.3	6		2	1		3
	1:1,000	.1	6	1	1	2		2
3	1:10,000	.15	6	1	1	1	2	1
	1:10,000	.5	6	3	3			
	1:1,000	.15	6	4	1			1
4	1:10,000	.1	10	1	2		3	4
	1:10,000	.2	10	2	3		3	2
	1:10,000	.4	10		4	2	3	1
5	1:10,000	.1	10		1			9
	1:10,000	.25	10		3	2	1	4
	1:10,000	.5	10	1	3	3	2	1
6	1:100,000	.4	10			2	1	7
	1:10,000	.1	10		1	2	2	5
	1:10,000	.3	10	3	2	1	3	

[1] These various concentrates contained about 10 mg. of the active fraction per cubic centimeter.
[2] The severity of induced hemorrhage is indicated by the number of plus signs. Tumors negative for hemorrhage are indicated by the minus sign.

with 10 mice per dose ideal results, such as those illustrated in experiment 6, were not often obtained, but the data were sufficiently consistent (as in experiment 5) for our purpose. For greater reliability, assays of important fractions were done 2 or more times.

Examination of Tumor Interior

For rough estimates of potency, simple inspection of the intact tumors was adequate, inasmuch as the tumors protruded from the anterior surface of the body and induced hemorrhage was readily evident. For more accurate estimates of the end point, however, some error is occasionally introduced when reliance is placed on observation of the intact tumors. With marginal doses, which produce hemorrhage in a small area of the neoplasm, the outer surface of the tumor may appear negative whereas the interior of the tumor may be affected. This is shown by comparative observations of macroscopic hemorrhage in intact tumors and in the same tumors when cut open. In table 2, the first four experiments illustrate the fact that intact tumors which appear negative may contain in the interior areas of induced hemorrhage not evident at the surface of the tumor. In all five experiments the severity of the hemorrhage, as judged by inspection of the intact tumors, was less than that evident on examination of the interior of the tumor.

This is not a source of serious error when large amounts of active material are given, for in such cases the surface portion is almost invariably affected, although less extensively in many cases than the interior. However, with borderline doses, it is a factor which should not be ignored, lest tumors with induced hemorrhage be classified as negative. The data given subsequently in this paper and in the following papers refer to observations of hemorrhage in the interior of the tumors.

Bacterial Contamination

Comparative experiments demonstrated that infected tumors may give a response different from that obtained with sterile tumor tissue, the infected tumors usually being more resistant. Table 3 gives illustrative results with 2 different concentrates, both of which contained about 10 mg. of the active fraction per cubic centimeter. As was usually the case, the concentrates had to be diluted 10,000 or 100,000 times so that doses near the end point might be contained in 0.1 to 0.5 cc.

Table 2.—*Comparative observations on hemorrhage, induced in sarcoma 37 by low doses of active preparations from* Serratia marcescens *culture filtrates, evident macroscopically in tumors before and after section*

Experiment [1]	Tumor inspection	Mice with induced hemorrhage in tumor				
		++++	+++	++	+	−
		Number	*Number*	*Number*	*Number*	*Number*
1	Intact				1	9
	Cut open		2	1	5	2
2	Intact		1	3	3	3
	Cut open	1	3	4	2	
3	Intact		1	2	3	4
	Cut open	1	5	2	1	1
4	Intact		2	2	1	
	Cut open	4	3	1	2	
5	Intact	1	3	3	3	
	Cut open	6	4			

[1] 10 mice were used in each experiment.

TABLE 3.—*Reaction of sterile and infected implants of sarcoma 37 to hemorrhage-production by minimal doses of the active fraction from* Serratia marcescens *culture filtrates*

Preparation	Tumor quality	Dose of concentrate: Dilution	Dose of concentrate: Volume injected	Mice used	Mice with induced hemorrhage in tumor: ++++	+++	++	+	−
			Cubic centimeter	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
B	Sterile	1:100,000	0.5	10			3	2	5
		1:10,000	.15	10			3	5	2
	Infected	1:100,000	.5	15					15
		1:10,000	.15	15			1	3	11
	Sterile	1:100,000	.5	10			1	5	4
		1:10,000	.15	10		1	4	3	2
C	..do	1:10,000	.1	10		1	2	2	5
		1:10,000	.3	10	3	2	1	3	1
	..do	1:10,000	.15	10			1	3	6
		1:10,000	.5	10	2	3	4		1
	Infected	1:10,000	.1	10					10
		1:10,000	.3	10					10

With preparation B, the first and third experiments were performed with tumor tissue that gave negative cultures for aerobic contaminants; the middle experiment was carried out with infected tumors. These experiments were done in 3 successive weeks. In both experiments with sterile tumor tissue, a dose of 0.5 cc. of 1 : 100,000 dilution induced hemorrhage in about 50 percent of the tumors, whereas a dose three times as large was definitely below the end point when infected tumors were used.

With preparation C, the experiments were carried out in 3 successive months. In the first 2 experiments, performed with sterile tumor tissue, a dose of 0.1 or 0.15 cc. induced hemorrhage in about 50 percent of the tumors, while a dose of 0.3 cc. failed to produce hemorrhage in any of 10 contaminated tumors. In the present work the presence of infection obviously contributed to the variability of the response to the active agent. Results such as these offered another explanation of the irregularities occasionally obtained in bioassays in earlier work.

REFERENCE STANDARDS

As a further check on the variability of response arising from undetermined causes, a standard active material was included in each set of assay experiments to provide a base of reference. From time to time, as the work progressed, the standard material was changed, but in each instance the end-point value of the new reference standard was established by repeated assays.

Preliminary experiments were usually performed to ascertain the approximate value of the end point; for this purpose a relatively small number of mice was used. Then the assay was carried out with 3 or more dose levels, differing usually threefold from one another. Since 10 mice were used for each dose, 30 tumor-bearing mice were required for each material assayed.

Table 4 illustrates the reproducibility obtained in three assays of a preparation (K–15b) which at one period of the work was used as a comparison standard material. This concentrate contained about

TABLE 4.—*Reproducibility of results in repeated assays of an active concentrate (K–15b) from* Serratia marcescens *culture filtrate*

Assay experiment	Dose injected		Mice with induced hemorrhage in tumor					End point
	Dilution	Volume	++++	+++	++	+	−	
		Cc.						
1	1 : 100,000	0.5			1	5	4	0.5 cc. of 1 : 100,000.
	1 : 10,000	.15		1	4	3	2	
	1 : 10,000	.5	4	2	3		1	
2	1 : 100,000	.5				3	7	0.1 cc. of 1 : 10,000.
	1 : 10,000	.15	1	2	1	3	3	
	1 : 10,000	.5	6	3		1		
3	1 : 100,000	.5			3	2	5	0.5 cc. of 1 : 100,000.
	1 : 10,000	.15			3	5	2	
	1 : 10,000	.5	6	2	1	1		
4	1 : 100,000	.3				2	8	0.1 cc. of 1 : 10,000.
	1 : 10,000	.1		1	1	3	5	
	1 : 10,000	.3	1	3	3	2	1	
	1 : 1,000	.1	3	4	1			

10 mg. of the active material per cubic centimeter. After establishing that the end-point dose was in the vicinity of 0.1 cc. of a 1 : 10,000 dilution, a more precise determination was carried out, with the results shown in experiment 1 of table 4. A dose of 0.5 cc. of 1 : 100,000 dilution produced hemorrhage in the tumors of about half of the animals. Larger doses gave hemorrhage in a progressively larger proportion of the tumors; the number of mice showing severe hemorrhage also increased with the dose. The end point in this experiment was therefore estimated to be about 0.5 cc. of 1 : 100,000 dilution.

In experiment 2 (table 4), this lowest dose gave 7 negative results and 3 weak positive ones, i. e., it was below the end-point dose. The next larger amount produced hemorrhage in 7 of 10 tumors. Hence, the end-point dose was intermediate between these 2 values and was estimated to be about 0.1 cc. of 1 : 10,000 dilution.

In experiment 3, the end-point dose was again about 0.5 cc. of 1 : 100,000 dilution. In experiment 4, it was found that 0.3 cc. of 1 : 100,000 dilution was well below the end point which in this case was 0.1 cc. of 1 : 10,000 dilution. The average value of the end point in the four determinations was about 0.8 cc. of 1 : 100,000 dilution.

Sometimes the variation in the end point obtained in repeated bioassays of the same material was much greater than that shown in table 4. In general, determinations of potency had a reproducibility estimated to be about $^{+100\text{ percent}}_{-50\text{ percent}}$ e. g., a preparation with an average value for the end-point dose of 0.2 microgram gave, in the individual assays, values which ranged between 0.1 and 0.4 microgram. Because of the variations in the biologic response, no elaborate method was employed for the calculation of the end-point values from the data; they were estimated from inspection of the data and were usually recorded by one significant figure. This procedure was found adequate for the purposes of these studies.

PROCEDURE ADOPTED

In the first place, precautions were taken to minimize contamination of the tumor tissue by bacteria. The dissection of the stock tumors, the preparation of the tumor mash, and the implantation into new hosts were carried out under aseptic conditions in a sterile room. Each batch of tumor tissue employed for transplantation

was tested for aerobic contaminants. When the cultures showed the presence of microorganisms, the mice which had received infected tumor tissue were not used in bioassays. It is not sufficient to discard only those mice in which pus is evident in the tumors or even to discard entire batches of mice in which gross infection is obvious in some tumors. Lesser degrees of contamination may give anomalous results in the assays.

Usually three, and sometimes four dose levels of the material under assay were injected intraperitoneally into mice bearing 6-to-8-day-old tumors growing just under the skin. Ten tumor-bearing mice were used for each dose. The animals were killed the day following the injection of the test material, and the tumors were cut open for examination of induced hemorrhage.

In each set of bioassays, data were obtained on the reaction of the tumors to a standard active preparation, the potency of which had been determined by repeated assay. Table 5 gives an illustrative set of bioassays performed in the way outlined. Preparation K–15c was subjected to two types of chemical manipulation. Preliminary assays had shown that one procedure (A) destroyed about half of the original potency, whereas the other (B) destroyed most of the potency. Hence, the dose range selected for the former was the same as for the untreated material, whereas a higher dose range was selected for the latter. Inasmuch as the untreated preparation (K–15c) had previously been assayed repeatedly with consistent results, it was employed here as the reference standard.

The standard gave an end point of 0.1 cc. of 1:10,000 dilution in agreement with the results of previous determinations. After manipulation A, the end point was just under 0.3 cc. of 1:10,000; this indicated that about 60 percent of the potency had been destroyed. After manipulation B, 0.1 cc. of 1:100 dilution was still considerably below the end-point dose; hence, more than 99 percent of the potency had been destroyed by this procedure. Determination of these few points required the use of 90 tumor-bearing mice in this set of bioassay experiments.

DISCUSSION

This method of bioassay was developed as a tool in the prosecution of the fractionation work described in the two papers following (*3*, *4*). In the simpler form originally employed, it was found useful for certain purposes. For the more precise determination of potency, however, and for discriminating between potencies which were relatively close together, the

TABLE 5.—*Sample set of bioassays of active concentrates from* Serratia marcescens *culture filtrate for potency in producing hemorrhage in mouse sarcoma 37*

Material assayed	Dose injected		Mice with induced hemorrhage in tumor					End point
	Dilution	Volume	++++	+++	++	+	−	
		Cc.						
Reference standard (K–15c).	1:100,000	0.4			2	1	7	0.1 cc. of 1:10,000.
	1:10,000	.1		1	2	2	5	
	1:10,000	.3	3	2	1	3	1	
K–15c after manipulation A.	1:100,000	.4			1	1	8	0.25 cc. of 1:10,000.
	1:10,000	.1		1	1	1	7	
	1:10,000	.3		2	3	1	4	
K–15c after manipulation B.	1:1,000	.1					10	>0.1 cc. of 1:100.
	1:1,000	.3				1	9	
	1:100	.1				2	8	

technique formerly used did not suffice, and it was found necessary to introduce modifications.

Increasing the number of mice used for each dose resulted, as expected, in a considerable improvement in the consistency of the data, as did substitution of examination of the interior of the tumors in lieu of inspection of the exterior surface. In spite of these modifications, the method was not sufficiently sensitive for the purpose desired, and anomalous results occurred too frequently. Testing the tumor mash for bacterial contamination and discarding all contaminated batches resulted in a further pronounced improvement in the consistency and reproducibility of the data.

Irregularities in the hemorrhagic response, originating from undetermined causes, still occurred occasionally even when the foregoing measures were taken. As a check on unusual resistance or sensitivity of an entire batch of tumors to the agent studied, a standard material of previously determined potency was therefore included in each set of bioassays. As a further precaution against freak irregularities, the assay of important materials was repeated two or more times with different batches of tumor-bearing mice.

Zahl and Hutner (*5*) have also employed a method based on the determination of the minimum dose which produces hemorrhage in the tumors of 50 percent of the animals. They designated this quantity the "minimum hemorrhage dose (M. H. D.)." It has the same significance as our "end-point dose," which we had designated (*6*, *7*) one "mouse tumor unit (m. t. u.)."

SUMMARY

The method formerly employed in the bioassay of bacterial preparations for potency in producing hemorrhage in mouse sarcomas was found unsuitable for work on further purification and on the chemical and physical properties of the active agent. Increasing the number of mice used for each dose, examining the interior of the tumors for hemorrhage, testing the tumor mash for bacterial contamination, and including a standard comparison material in each set of bioassays yielded data of greater consistency and reproducibility.

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Chemical Treatment of Tumors. VII. Nature of the Hemorrhage-Producing Fraction From *Serratia marcescens (Bacillus prodigiosus)* Culture Filtrate[1]

By J. L. HARTWELL, *chemist*, M. J. SHEAR, *principal biochemist*, and JOHN R. ADAMS, JR., *assistant chemist*, with the technical assistance of ADRIEN PERRAULT, *National Cancer Institute, National Institute of Health, United States Public Health Service*

A previous communication (*2*) from these laboratories described the preparation of a polysaccharide fraction from filtrates of *Serratia marcescens* (*Bacillus prodigiosus*) cultures, which was highly potent in producing hemorrhage in mouse sarcomas. The present paper reports the further purification of the product and the results obtained to date on the nature and composition of the material as revealed by chemical degradation experiments.

Highly potent concentrates of the active polysaccharide fraction (*2*) were submitted to tryptic digestion. This treatment lowered the nitrogen content from about 4 to 2 percent without detectable loss of potency. With the trypsin-digested material, hemorrhage was produced in 50 percent of the tumors by a dose of 0.1 microgram. About two-thirds of this material consisted of sugar residues, of which the major portion was aldohexose. The active material, after tryptic digestion, also contained small amounts of phosphoric acid, a solid fatty acid, glycerol, and a nitrogenous constituent, i. e., the components of a phospholipid. The lipid was present in firm combination and, up to the present, methods which removed the lipid also destroyed the potency. No evidence was found for the presence of protein or polypeptide.

[1] *Presented in part* (1) *at the Boston meeting of the American Association for Cancer Research, March 31, 1942.*

MATERIALS AND METHODS

STARTING MATERIAL

The concentrates prepared in the previous work were turbid, viscous solutions, neutral in reaction. The preparations had been dialyzed in cellophane bags against water and therefore contained only nondialyzable constituents. Lyophilization of these concentrates gave a light gray, fluffy, noncrystalline product which was readily soluble in water.

The aqueous solutions gave negative tests for proteins by the biuret, ninhydrin, xanthoproteic, and Millon's reactions. The solid material also reacted negatively to the Millon test. The Molisch test for polysaccharide was strongly positive. The Benedict test for reducing sugars was negative, but after short boiling of the concentrate with dilute hydrochloric acid it was positive. The iodine-potassium iodide test was negative, showing the absence of such substances as starch, glycogen, and certain dextrins.

These concentrates gave precipitates with several reagents. Phosphotungstic acid and silver nitrate each gave a sediment within 3 hours; flavianic acid, trichloracetic acid, and sulfosalicylic acid yielded sediments only after a longer time; and tannic acid and potassiomercuric iodide gave only turbidities. Picric acid, picrolonic acid, and Reinecke salt

gave no precipitate. These preparations therefore contained only small amounts of proteinaceous materials, although the nitrogen content was about 4 percent.

The active material contained about 6 percent "acetyl," which was not identified as such but represented the volatile acids calculated as acetic acid. Ash was present to the extent of about 3.5 percent. Only 0.16 percent sulfur was found. This constituent was not in a labile or unoxidized form, as shown by testing with lead acetate after boiling with dilute sodium hydroxide. The molybdate test for inorganic phosphate was negative, but elementary analysis gave a value of 1 percent phosphorus.

Bioassay Method

Assays of potency in producing hemorrhage in tumors were carried out according to the technique described in the preceding paper (*3*). Each step in the manipulations was correlated with its effect on the potency in this way. In addition, assays of important steps were performed two or more times for greater reliability.

RESULTS

Further Purification by Tryptic Digestion

The partially purified starting material, which was chiefly polysaccharide in nature, was submitted to the action of a number of proteolytic enzymes for 2 days at 37° C. Trypsin was employed at pH 8.5, pepsin at pH 2.1, and papain at pH 4.4. As judged by analysis of the dialyzates for tyrosine,[2] trypsin was the only one which gave evidence of digestive action.

Treatment of the active polysaccharide fraction with amylopsin (pancreatic amylase) at pH 6.9 for 24 hours at 37° did not split off reducing sugars, nor was any change in the tyrosine value obtained. The potency of the material remained unaltered. The action of this carbohydrate-splitting enzyme was therefore not studied further.

In the tryptic digestion, the concentrate was treated with 0.008 of its dry weight of trypsin[3], brought to pH 8.5 by the addition of phosphate buffer, and set aside (under toluene) at 37° C. for at least 2 days. The solution, which had become much less turbid, was then dialyzed against running water and precipitated by alcohol alternately from alkaline (pH 9.5) and acid (pH 2.0) solution. The final precipitate was dissolved in water and dialyzed again.

Within wide limits of trypsin excess, the tyrosine value of the nondialyzable material could not be reduced below a certain minimum figure; this apparently represented a reducing action of the polysaccharide itself on the phosphomolybdotungstate reagent, since *d*-glucose and *d*-glucosamine hydrochloride were found to give reduction corresponding to 0.09 and 0.20 percent, respectively, of their weight of tyrosine. The buffer alone, in the absence of trypsin, was ineffective in liberating tyrosine. Furthermore, progressive addition of trypsin to the concentrate, with or without intermediate removal of degradation products by dialysis, failed to lower the tyrosine value below this minimum. On previously tryptic-digested material, trypsin had no further action that was apparent.

After tryptic digestion, the material gave entirely negative results with most of the reagents for proteins or polypeptides that had been applied to the starting material. (See p. 107.) A small amount of sediment was obtained with trichloracetic acid, with

[2] *By the method outlined in Koch's manual* (4, p. 248).

[3] *Trypsin 1 : 250 (Difco Laboratories). This was a crude extract of pancreas which was not purified further since it was considered desirable to retain enzymes other than trypsin itself.*

potassiomercuric iodide, and with silver nitrate; however, these sediments were obtained only on long standing. The tryptic-digested material was also tested with several additional reagents: a turbidity was obtained with barium hydroxide and with neutral lead acetate; copper sulfate, and mercuric chloride both gave negative results.

The trypsin-digested material was optically active with a specific rotation $[\alpha]_D^{20}$ of $+128°$. The determination was made on an approximately 1-percent neutral aqueous solution in a 0.254-dm. cell.

Representative analyses [4] of various lots of concentrate before and after tryptic digestion are given in table 1. Digestion produced significant changes in two respects: (1) The nitrogen content was lowered from about 4 to about 2 percent; and (2) the "acetyl" content was lowered from about 6 to about 2 percent. No methoxyl was found on analysis of the trypsin-digested material.

Treatment with trypsin had no detectable effect on the potency of the active material. Three portions of preparation K–15 were digested separately, at different times. The activity of these products was compared with that of undigested K–15 in 4 assay experiments (table 2). In each of the first 2 sets of experiments the comparative assays were carried out simultaneously; 60 mice of the same batch of tumor-bearing animals were used in each experiment. The other 2 assays were performed on different days with tumor-bearing mice of different batches. The assays showed that treatment with trypsin did not diminish the activity.

All the data reported henceforth in this paper were obtained with trypsin-digested material.

Action of Heat

The solid material or the dilute aqueous solution could be preserved indefinitely at 10° C. with no loss of potency. The solution showed no appreciable change in activity when kept at 37° for several weeks. In the temperature range 60°–100° the activity rapidly decreased with time. Loss of potency at 60° was noticeable in 1 hour, while at 100° there was complete destruction within one-half hour. Table 3 gives the data on the loss of potency when neutral solutions of the active concentrate in test tubes were immersed in the vapors of various boiling liquids for ½ hour to 16 hours.

Heating of the neutral aqueous solution resulted in the separation of a brownish

[4] *The microanalyses were performed by the Arlington Laboratories, Chagrin Falls, Ohio, unless otherwise noted.*

TABLE 1.—*Composition of the hemorrhage-producing polysaccharide fraction,*[1] *from* Serratia marcescens *culture filtrate, before and after tryptic digestion*

Sample	Tryptic digestion	C	H	N	P	Acetyl	Ash
		Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
K–8d	Before	47.6	7.3	3.8	0.9	7.1	3.8
K–15	do			4.3		4.6	3.2
K–15	After			2.1		2.4	
K–15b	do	48.6	7.2	2.1		2.2	3.3
K–15c	do				1.1	3.0	
P–2	do	46.6	7.0	1.6	1.0	2.2	[2] 5.3
P–3	do	47.4	7.0	2.9	1.1	1.2	3.8
Average	After	47.5	7.1	2.2	1.1	2.2	3.5

[1] The solutions were evaporated to dryness and the residues dried to constant weight for analysis.
[2] Not included in average.

TABLE 2.—*Bioassays showing the resistance to tryptic digestion of the hemorrhage-producing activity of the polysaccharide fraction from* Serratia marcescens *culture filtrate*

Specimen of concentrate	Tryptic digestion	Volume of 1:10,000 dilution injected	Mice with induced hemorrhage in tumor					End-point dose [1]
			++++	+++	++	+	−	
		Cubic centimeter	*Number*	*Number*	*Number*	*Number*	*Number*	*Cubic centimeter*
K-15	Before	0.1			1	2	7	0.15
		.25	5	3			2	
		.5	7	2	1			
	After	.1		1	2	1	6	.1
		.25	4	3	2	1		
		.5	6	2	2			
K-15a	Before	.1	1	3	1	1	4	.1
		.2	4	3	1		2	
		.4	6	1	1	1	1	
	After	.1		3			7	.15
		.2	5	4			1	
		.4	3	5			2	
K-15b	Before	.1		1			9	.25
		.25		3	2	1	4	
		.5	1	3	3	2	1	
	After	.1	1	2		3	4	.1
		.2	2	3		3	2	
		.4		4	2	3	1	

[1] 1 : 10,000 dilution.

substance, insoluble in water but soluble in alkali.

ACTION OF ACID

Solutions of the active material were brought to pH 5, 3, and 1 by means of hydrochloric acid and kept for 24 hours at room temperature; they were then dialyzed against distilled water. The dialyzates were all found to be inactive while the nondialyzable fractions retained the original potency in full.

When submitted to the action of 2 N hydrochloric acid at 37° C., the activity was rapidly destroyed, about 90 percent being lost in 6 hours (table 4). At 48 hours, less than 1 percent of the potency remained; more than half of the dissolved material was now dialyzable. The nondialyzable residue, on analysis, yielded only 0.75 percent acetyl as compared with a value of 3.0 percent for this preparation prior to treatment with acid.

TABLE 3.—*Rate of destruction with heat of the hemorrhage-producing potency of the polysaccharide fraction from* Serratia marcescens *culture filtrate.*

Temperature (in degrees centigrade)	Mice used in assays	Time required for loss in potency of—	
		50 percent	90 percent
	Number	*Hours*	*Hours*
60	151	4	>16
80	130	2	8
100	103	<0.5	1

TABLE 4.—*Rate of destruction with 2 N hydrochloric acid at 37° C. of the hemorrhage-producing potency of the polysaccharide fraction from* Serratia marcescens *culture filtrate*

Temperature (in degrees centigrade)	Mice used in assays	Residual potency after various periods					
		1.5 hours	3 hours	6 hours	12 hours	24 hours	48 hours
	Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
37	517	>50	>50	10	5	2	<1
80	30	0					

The progress of the inactivation by hydrochloric acid was followed with simultaneous determinations of reducing sugar,[5] hexosamine,[6] and inorganic phosphorus [7] (table 5). The amounts of these compo-

[5] *By the Hagedorn-Jensen-Hanes method* (5).
[6] *By the method of Palmer, Smyth, and Meyer* (6).
[7] *By the method of Youngberg, as given by Hawk, Bergeim, Oser, and Cole* (7, p. 454).

TABLE 5.—*Rate of hydrolysis of the active polysaccharide fraction from* Serratia marcescens *culture filtrate with 2 N hydrochloric acid at 37° C.*

Time (in hours)	Reducing sugar [1]	Hexosamine	Phosphorus
	Percent [2]	*Percent* [3]	*Percent* [4]
0	4.1	2.4	5.3
¾	4.7	4.2	6.3
1½	6.8	7.5	13.3
3	7.3	6.0	17.0
6	8.8	5.3	19.5
12	14.9	10	25
24	25.2	19	30
48	38.0	38	32

[1] Calculated as glucose.
[2] Based on 64 percent total glucose obtained on maximum hydrolysis.
[3] Based on 2.0 percent total hexosamine obtained on maximum hydrolysis.
[4] Based on 1.1 percent total phosphorus obtained on elementary analysis.

nents liberated were smaller than anticipated; however, they all increased progressively as the potency decreased.

The rates of destruction of potency and of liberation of reducing sugar, of hexosamine, and of inorganic phosphate are shown in figure 1. The data are given in percentages, for each component, of the amount liberated in 48 hours, after deduction of the values for the blanks, i. e., the values obtained at zero time. Hydrolysis released reducing sugars and hexosamine at rates which were approximately proportional to the time, and which did not parallel the rate of loss of potency. The hydrolysis curve for phosphate, however, exhibited a sharp rise during the first 12 hours as did the curve for destruction of potency. Whether there is a significant relationship between loss of phosphate and loss of potency remains for further investigation to determine.

The optimum concentration of hydrochloric acid for maximum hydrolysis at 100° was found to be 1.5 N, when 64 percent of reducing sugars (calculated as glucose) was released in 3 hours (fig. 2).

At 80°, 2 N hydrochloric acid caused complete inactivation within 1.5 hours (table 4) along with the separation of a light gray, flocculent precipitate, which was soluble in dilute alkalies. From the alkaline solution the precipitate could be produced again by acidification. At 37° a similar precipitate was obtained on standing for a month. Further data on this component are given on page 115.

ACTION OF ALKALI

Solutions of the concentrate were brought to pH 9 and 11 with sodium hydroxide and dialyzed against water at room temperature for 24 hours; no loss of potency occurred in the nondialyzable fraction. Treatment with 0.1 N sodium

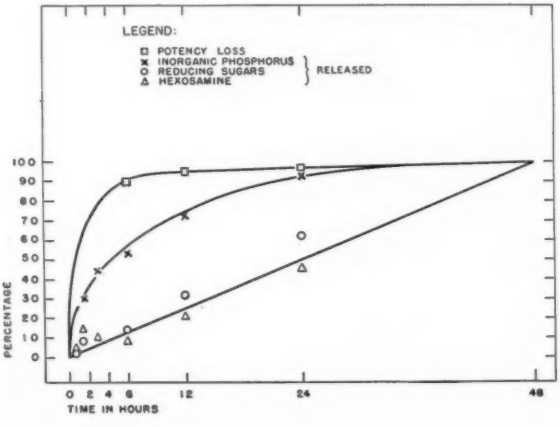

FIGURE 1.—Effect of 2 N hydrochloric acid at 37° C. on the active polysaccharide fraction from *Serratia marcescens* culture filtrate. The curves represent the rate of destruction of potency and the rates of liberation of three components.

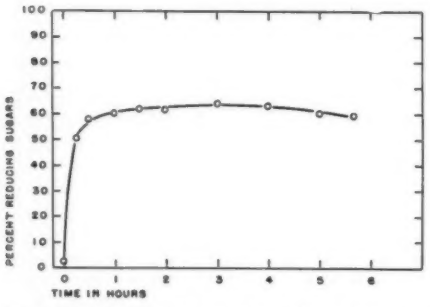

FIGURE 2.—Rate of hydrolysis of the active polysaccharide fraction from *Serratia marcescens* culture filtrate with 1.5 N hydrochloric acid at 100° C.

hydroxide at room temperature (28° C.) resulted in destruction of about two-thirds of the potency in 4 hours and of about nine-tenths in 12 hours (table 6); the solution turned light amber in color, and the turbidity cleared rapidly. No significant amount of material was lost on dialysis, but the nondialyzable solid on analysis showed a complete loss in "acetyl"; analyses of other components showed no significant change.

At 37° about two-thirds of the potency was lost in 3 hours (table 6). At 56° about 90 percent of the activity was destroyed in 1 hour; concomitantly the acetyl content fell to zero while the nitrogen content was unchanged. At 60°, about three-fourths of the activity was destroyed in 15 minutes, and almost all was gone in 1 hour.

The progress of inactivation with 0.1 N sodium hydroxide at 37° was followed with simultaneous determinations of reducing sugar, hexosamine, and phosphoric acid; the amounts of these substances liberated were found (table 7) to be much smaller than was the case with 2 N hydrochloric acid.

The action of 0.1 N sodium carbonate at 37° C. was similar to that of sodium hydroxide but less drastic. After 24 hours, about four-fifths of the activity was destroyed; simultaneously the greater part of the acetyl was removed from the nondialyzable fraction while the nitrogen content remained essentially the same (table 8). The effect of 10 percent by volume of pyridine, acting for 24 hours at 37° C. was to destroy about half the activity; about two-thirds of the acetyl was removed, while the nitrogen content was essentially unchanged. The only action of 0.1 N sodium bicarbonate was the reduction of the acetyl to the lowest values obtained with full potency, viz, 0.6 and 1.2 percent. After treatment for 45 hours with sodium bicarbonate under these conditions, potency was not decreased while the nitrogen and phosphorus values remained unchanged.

TABLE 6.—*Rate of destruction of potency of the active polysaccharide fraction from* Serratia marcescens *culture filtrate by treatment with 0.1 N sodium hydroxide at different temperatures*

Temperature (in degrees centigrade)	Mice used in assays	Residual potency after various periods					
		1 hour	1½ hours	3 hours	4 hours	7 hours	12 hours
	Number	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*	*Per-cent*
28	250	.....	.....	100	30	.....	[1] 10
37	330	.....	100	30	.....	15	.....
56	30	[1] 10	.....	.....	.....	.....	.....
60	90	<1	.....	.....	.....	.....	.....

[1] "Acetyl" content reduced to zero.

TABLE 7.—*Rate of hydrolysis of the active polysaccharide fraction from* Serratia marcescens *culture filtrate with 0.1 N sodium hydroxide at 37° C.*

Time (in hours)	Reducing sugar [1]	Hexosamine	Inorganic phosphorus
	Percen [2]	*Percent* [3]	*Percent* [4]
0	2.8	2.2	5.0
¾	2.8	4.2	4.8
1½	3.1	5.7	4.8
3	3.2	5.8	5.1
7	3.6	10.9	5.4
12	3.5	5.4	5.7
24	4.1	7.8	5.4

[1] Calculated as glucose.
[2] Based on 64 percent total glucose obtained on maximum hydrolysis.
[3] Based on 2.0 percent total hexosamine obtained on maximum hydrolysis.
[4] Based on 1.1 percent total phosphorus obtained on elementary analysis.

TABLE 8.—*Effect of treatment of the active polysaccharide fraction from* Serratia marcescens *culture filtrate with weak alkalies for 24 hours at 37° C.*

Alkali	Mice used in assays	Residual potency	Nitrogen content		Acetyl content	
			Before	After	Before	After
	Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Sodium carbonate [1]	100	20	2.1	1.9	3.0	0.2
Pyridine [2]	45	50	2.1	1.9	3.0	.9
Sodium bicarbonate.[3]	145	{100	2.1	------	3.0	.6
		{100	1.6	1.5	2.2	1.2

[1] 0.1 N.
[2] 10 percent by volume.
[3] These data were obtained in 2 sets of experiments with different preparations of active material.

Qualitative Identification of Sugar Components

In this work the scheme of Militzer (*8*) was found to be useful. Unless otherwise specified, the tests were carried out according to the directions given by Militzer or by Van der Haar (*9*). The lyophilized solid, the aqueous solution, and the hydrolyzate prepared by heating the active material at 100° C. with 1.5 N hydrochloric acid were all used in the tests. For controls, appropriate sugars were used.

The polysaccharide did not reduce Benedict's reagent, whereas the hydrolyzate was strongly reducing. The following tests all gave negative results: the iodine-potassium iodide test for starch, glycogen, and dextrin; Seliwanoff's resorcinol test for ketone sugar; Bial's orcinol and the Wheeler-Tollens phloroglucinol tests for pentose and uronic acid; the aniline and the Bayer-Rosenthaler resorcinol tests for pentose; and Kiliani's test for desoxy sugar. The negative results of the Bial test also indicated the absence of heptose.[8] The foregoing tests ruled out the presence of most sugars except hexoses and methylpentoses. The Rosenthaler and the Oshima-Tollens phloroglucinol tests for methylpentose, and the Elson-Morgan test (*10*) for amino sugar were strongly positive.

[8] *Private communication from Dr. C. S. Hudson.*

The latter was not an unusual finding, for *d*-glucosamine has been reported several times as occurring in bacterial polysaccharides. Inositol was shown by the Salkowski modification of the Scherer reaction (*11*) to be absent.

Since quantitative data indicated the presence of a large amount of reducing sugar which was not accounted for as methylpentose or as amino sugar, the presence of a considerable amount of aldohexose was indicated. Qualitative tests showed the absence of the two aldohexoses other than glucose which are commonly encountered in bacterial polysaccharides. Thus, the mucic acid test for galactose and the phenylhydrazone test for mannose were both negative. The hydrolyzate nevertheless yielded a crystalline osazone product which under the microscope was seen to be a mixture of yellow needles and orange plates; the yellow needles bore a strong resemblance to a specimen of glucosazone prepared under similar conditions. The aldohexose was therefore provisionally regarded as glucose. Further chemical work on identification of the individual sugars present had to be postponed because of factors beyond our control.

Quantitative Determination of Sugar Components

The determination of methylpentose was carried out by the Krüger-Tollens-Kröber method (*9*). From a solution of concentrate containing 214 mg. of polysaccharide, 18.1 mg. of phlorogluciде was obtained. According to the tables of Ellett and of Mayer, as given by Van der Haar (*9*), this corresponds to 35.4 mg. of anhydrous rhamnose, or 44.6 mg. of fucose, i. e., to 16.5 or 20.8 percent, respectively. The phenylhydrazone test applied to the hydrolyzate failed to yield the precipitate which fucose gives. Whether the methyl-

pentose was rhamnose or fucose was not established, but the available information favored the former of these two commonest representatives of their class.

Aminohexose was determined by the colorimetric method of Palmer and coworkers (*6*). With two different batches of material, values of 3.3 and 2.0 percent, calculated as *d*-glucosamine, were obtained. As far as we are aware, no aminohexose other than *d*-glucosamine has been identified in bacterial polysaccharides.

The amount of aldohexose that is formed on hydrolysis may be estimated from the value of 64 percent found for the total reducing sugars (calculated as glucose), less the determined amounts of methylpentose and aminohexose after conversion to their respective glucose equivalents based on their reducing power. By using pure dry samples of the sugars, it was found by the Hagedorn-Jensen-Hanes method that 1 mg. of anhydrous rhamnose, 1 mg. of fucose, and 1 mg. of *d*-glucosamine gave glucose equivalents of 1.04, 0.80, and 1.00 mg., respectively.[9] Under similar conditions of hydrolysis, rhamnose suffered no loss in reducing power, while *d*-glucose lost only 6.7 percent. Thus, in the hydrolyzate, 16.5 percent of rhamnose would appear as 17.2 percent when calculated as glucose while the value of glucosamine (2.7 percent average) would appear unchanged. Subtraction of the sum of these two values from 64 percent gave 44.1 percent which would represent the content of *d*-glucose. If fucose is assumed to be the methylpentose present, the percentage of glucose would be 44.7.

Some support for the assumption made regarding the identity of the component sugars is given by data on optical rotation. After hydrolysis under optimum conditions, the observed optical rotation, $[\alpha]_D^{20}$, changed from + 128° to + 36°. The calculated values for the optical rotation of mixtures of these sugars, in the relative proportions postulated, agreed well with the observed rotation for the hydrolyzate when rhamnose was assumed to be the methylpentose (table 9). When fucose was taken as the methylpentose, the agreement was much less satisfactory.

Detection of Components Other Than Sugars

Nitrogen comprised 2 percent of the trypsin-digested polysaccharide fraction. The 2.7 percent of hexosamine accounted for 10 percent of the total nitrogen. On hydrolysis an alkali-soluble product was obtained which resisted further hydrolysis; it contained 1.9 percent nitrogen. This material appeared to be similar to the "humic acids" observed as byproducts in the hydrolysis of other polysaccharides (*13*).

Heat alone also caused the separation

Table 9.—*Comparison of the observed optical rotation of the hydrolyzate of the active polysaccharide fraction, from* Serratia marcescens *culture filtrate, with values calculated for mixtures of different simple sugars*

Mixture	Sugar	[α] of simple sugars[1]	Composition of mixture	[α] calculated[2]	[α] of hydrolyzate, observed
		Degrees	*Percent*	*Degrees*	*Degrees*
A	*d*-Glucose	+52.5	69.7	+36.6	
	Rhamnose[3]	+ 9.9	26.1	+ 2.6	
	d-Glucosamine	+44	4.2	+1.8	
Total			100.0	+41.0	+36
B	*d*-Glucose	+52.5	65.5	+34.4	
	Fucose	−75.6	30.5	−23.0	
	d-Glucosamine	+44	4.0	+1.6	
Total			100.0	+13.0	+36

[1] Experimental values taken from the literature.
[2] Obtained by multiplying values in column 3 by those in column 4.
[3] Anhydrous.

[9] *This last value is different from that found by Freudenberg and Eichel* (12), *who reported that glucosamine has only 70 percent of the reducing power of glucose as determined by the Hagedorn-Jensen method.*

of the component which was found to be soluble in alkali.

A quantity of the insoluble product was prepared by hydrolyzing a solution of the concentrate, containing 1.22 gm. of solid, with 1.5 N hydrochloric acid for 2.5 hours at 100°. The gray product, which felt greasy, was obtained in a yield of 0.191 gm. (or 16 percent). It gave a strong Molisch test for carbohydrate and a negative Liebermann-Burchard test for cholesterol. After treating it further with boiling alcoholic hydrochloric acid (equal volumes of 95 percent alcohol and concentrated acid) which apparently had not effected further hydrolysis, a light-colored solid was extracted with ether. This latter solid dissolved in alkali and precipitated again on addition of acid to the alkaline solution. It thus appeared to be a solid fatty acid. In the quantitative methylpentose determination, in which the concentrate was distilled with 12 percent hydrochloric acid, a white solid acid was also obtained, which was present in the steam distillate in about 4-percent yield.

The hydrolyzate, after removal of the acid-insoluble material, gave a strongly positive molybdate test for phosphoric acid. A portion of this solution, when evaporated to dryness and heated with dry potassium bisulfate, gave a strong odor of acrolein; this indicated the presence of glycerol.

The filtered hydrolyzate was analyzed for ammonium nitrogen and total nitrogen [10] and was tested for various nitrogenous substances. Only traces of ammonium nitrogen and of amino acids were found. The biuret test gave ambiguous results, and the Millon test was negative. The Folin-Looney phosphomolybdotungstate test for tyrosine, the Sakaguchi test for guanidines (arginine), and Ehrlich's diazo test were all positive. Benedict's modification of the Hopkins-Cole reaction for tryptophane, the Sullivan test for cystine, the bromine test for histidine, the Ehrlich test for pyrroles, and the murexide test for purines were all negative. The absence of purines was confirmed by Dr. Kahler, of the National Cancer Institute staff, who found in the unhydrolyzed concentrate no specific absorption in the ultraviolet region at the wavelength characteristic of purines.

Of the 2 percent total nitrogen in the polysaccharide fraction, 0.5 percent was accounted for by hexosamine and the alkali-soluble hydrolysis product. The remaining 1.5 percent of the total nitrogen was not accounted for quantitatively. Qualitatively, it was shown that the polysaccharide contained materials which gave positive colorimetric tests for certain amino acids even after tryptic digestion. The presence of a solid fatty acid, phosphate, glycerol, and soluble nitrogenous material indicated the presence of a phospholipid.

Elementary analysis of the polysaccharide fraction gave about 1 percent phosphorus and about 0.2 percent sulfur. The molybdate test for inorganic phosphate was negative prior to hydrolysis but was strongly positive in the clear, filtered hydrolyzate, showing that the phosphorus in the polysaccharide was present in organic combination. Not all of it, however, was released on hydrolysis, for the insoluble material obtained on digestion with acid still contained the same proportion of phosphorus as the original material.

With barium chloride, the clear hydrolyzate readily gave a small amount of precipitate, showing the presence of sulfate in traces.

[10] *By micro-Kjeldahl digestion, and determination of the ammonia colorimetrically with Nessler's reagent.*

Attempts to Separate the Polysaccharide and Phospholipid Moieties

To determine whether the polysaccharide and phospholipid components were united in a molecular complex or were present merely as a mixture, and in an attempt to ascertain which component was the seat of the hemorrhage-producing activity, several different types of experiments were carried out.

Morgan and Partridge (*14*) found that the polysaccharide moiety of a complex isolated from a dysentery bacillus could be separated from the phospholipid component by simple solution of the complex in anhydrous formamide and precipitation of the polysaccharide with alcohol. We found that lyophilized active preparations from *Serratia marcescens* dissolved readily in formamide to give a solution clearer than the aqueous one. Unlike the finding of Morgan and Partridge, however, addition of absolute alcohol to the formamide solution brought down the original product apparently unchanged; the hemorrhage-producing potency and the reducing value were essentially unaltered, and no appreciable lowering of the phosphorous content was effected.

The possibility that the phospholipid had been dissociated in formamide solution but re-entered the complex after addition of alcohol was tested in two ways. The formamide solution was dialyzed against formamide for 27 days, after first establishing that a formamide solution of lecithin would lose lecithin under these conditions. The formamide solution was also extracted with hexane. Neither of these procedures resulted in the removal of a phosphorus-containing component from the complex.

Fractional adsorption was also tried. The complex was shaken with successive additions of an adsorbing agent and was centrifuged after each addition; the supernatant solution was examined for phosphorus, nitrogen, and for biologic activity after each treatment. The adsorbing agents used were Lloyd's reagent, activated alumina, bentonite, norite B, calcium carbonate, and lead phosphate. There was no apparent adsorption of phosphorus or nitrogen or of the potent component with Lloyd's reagent, activated alumina, calcium carbonate, or lead phosphate. Bentonite removed most of the activity along with a large proportion of the nitrogen and the phosphorus. Norite B, however, removed most of the activity, but none of the phosphorus, and little of the nitrogen. Attempts to recover the potent material from the norite by elution with water, with 0.001 N hydrochloric acid, or with 0.001 N sodium hydroxide were unsuccessful. The experiments with norite indicated, however, that further work with adsorbents might be fruitful.

A sample of the concentrate was extracted continuously with hexane (b.p.65°–67°) at a temperature of 28°–38° C. On the ninth day it was made 0.1 N with respect to sodium bicarbonate, and the extraction continued for 6 more days. On the fifteenth day enough hydrochloric acid was added to neutralize the bicarbonate and to bring the solution to 0.1 N with respect to the acid; the extraction was then continued for 26 more days. Samples removed at intervals showed no significant reduction in phosphorus. This was good evidence that the phospholipid was not present in simple mixture. Another aliquot of the concentrate was brought to pH 11 by the addition of sodium hydroxide and glycine, and extracted continuously with hexane for 24 days; about one-half of the phosphorus was removed. It had previously been found (p. 111) that treatment of the complex for 24 hours at pH 11 did not produce any detectable diminution in potency. Hence the removal by hexane

at this pH of a phosphorus-containing component may possibly provide a method of separation of the type sought. No data were obtained on the activity of these fractions, for the investigation had to be discontinued at this stage.

MISCELLANEOUS EXPERIMENTS

In addition to the systematic work described in the foregoing sections, a number of miscellaneous experiments of an exploratory nature were carried out.[11]

Because of the sharp decrease in the "acetyl" content which usually accompanied loss of potency on hydrolysis, a few test experiments were performed from the standpoint of acetylation and de-acetylation. Several preparations were treated with acetic anhydride at room temperature, but no increase in potency was effected. Hydrolysis with 0.1 N sodium hydroxide at room temperature for 22 hours destroyed most of the potency; treatment of this product with acetic anhydride, likewise at room temperature, did not restore the potency to the original value. The partial de-acetylation procedure of Zemplén (*15*) was applied to the latter material; this also was ineffective in restoring the original activity.

Inasmuch as hexosamine was found to be a component of the polysaccharide, *d*-glucosamine in doses up to 5 mg. was injected into mice bearing sarcoma 37; no hemorrhage was produced in the tumors. *N*-acetyl-*d*-glucosamine, in doses up to 0.5 mg., likewise gave negative results.

Other carbohydrate substances which were found ineffective in producing hemorrhage in tumors were: *i*-inositol; α, *d*-galacturonic acid; glycuronic acid; trehalose; dihydroxyacetone; soluble starch; inulin; pectin; and α- and β-lignin sulfonic acids.

A number of hemolytic agents, anticoagulants, and capillary constrictors or dilators, were tested in tumor-bearing mice. Several substances related to these in structure or in physiologic action were also tested. Intraperitoneal or subcutaneous injection of the following materials gave negative results as regards production of hemorrhage in sarcoma 37 (the values in parentheses give the maximum dose injected):

Cholic acid (0.5 mg.); desoxycholic acid (0.5 mg.); sodium taurocholate (0.5 mg.); sodium glycocholate (0.2 mg.); sodium fluoride (0.2 mg.); sodium oxalate (0.2 mg.); sodium citrate (0.2 mg.); hirudin (0.5 mg.); Pontamine Sky Blue 6BX (0.5 mg.); Congo red (0.5 mg.); 3,3′-methylene-bis-(4-hydroxycoumarin) (0.5 mg.); histamine (0.2 mg.); penicillin (0.1 mg.); actinomycin (0.005 mg.); digitalin (0.5 mg.); peptone (0.2 mg.); and acetylcholine bromide (0.2 mg.).

Evidence of hemorrhage production in tumors was obtained with a few of the materials tested by intraperitoneal injection. Saponin, in a dose of 0.1 mg., killed half the mice but produced a moderate amount of hemorrhage in all the tumors; smaller doses gave negative results. Digitonin, in a dose of 0.5 mg., produced hemorrhage in the tumors of one-third of the animals. Three azo dyes, which were stated in the literature to be anticoagulants, were tested. The two which gave negative results are listed above; Pontamine Fast Pink BL, however, produced a moderate amount of hemorrhage in tumors in a dose of 0.5 mg. Adrenaline hydrochloride was lethal in doses of 0.1 mg. or more; 0.05 mg. was tolerated and produced a moderate degree of hemorrhage in some of the tumors. An old, weak, commercial preparation of heparin from dog liver produced hemorrhage in some of the tumors in doses of 0.5 and 2.0 mg. Four relatively fresh preparations of

[11] *In part in collaboration with Dr. F. C. Turner.*

heparin were then tested. Two specimens derived from beef lung were obtained from a commercial source. Two other specimens obtained from the Connaught Laboratories in Canada were stated to be highly purified preparations derived from beef lung and from sheep (organ not stated). In doses of about 1 mg., negative results were obtained with all four specimens of heparin. Fresh beef liver and lung were fractionated in this laboratory according to the methods employed for preparation of crude heparin; all fractions yielded negative results in tests for hemorrhage-producing potency. One possible explanation for the positive result given by the first heparin preparation is that it may have been contaminated, at some stage in its history, with bacteria possessing ability to produce hemorrhage in tumors.

However, Antopol and Glick (*16*) obtained evidence of preparatory and provocative potency, for the Shwartzman phenomenon in normal rabbit skin, in the case of sterile preparations from certain mammalian organs. Four of these preparations were obtained from Dr. Antopol and tested in mice bearing sarcoma 37. The dry specimens were dissolved in water and injected intraperitoneally in doses of 0.2 and 0.02 mg. One material gave negative results, and two others each produced a slight amount of hemorrhage in one of five tumors. The extract of steer pancreas, however, possessed considerable potency, for 0.2 mg. produced hemorrhage in all the tumors, and in two of the five mice the hemorrhage was severe. Even with 0.02 mg., hemorrhage was produced in some of the tumors. The pancreas employed had been cultured and found to be sterile both aerobically and anaerobically.[12] Antopol and Glick (*16*) stated that every step in the preparation of the fractions was of such a nature that sterility was automatically maintained. The positive result with this material warrants further investigation of mammalian tissue extracts.

An experiment was carried out to determine whether the bacterial polysaccharide possessed any hemolytic power. Washed rabbit erythrocytes were suspended in isotonic phosphate buffer solution (pH 6.7) containing the active polysaccharide in concentrations ranging from 0.001 to 1.0 mg. per cubic centimeter. A 0.9-percent solution of sodium chloride, and the same buffer solution containing saponin in the same concentrations as the polysaccharide, were employed as controls. Hemolysis was produced rapidly with concentrations of saponin down to 0.03 mg. per cubic centimeter, and progressively more slowly with lower concentrations down to 0.008 mg. per cubic centimeter. In contrast, no hemolysis was obtained with the polysaccharide solutions in any of the dilutions tested. The highest concentration of polysaccharide employed contained 10,000 mouse tumor units per cubic centimeter, i. e., 10,000 times the dose required to produce hemorrhage in 50 percent of the tumors. This indicated that the mechanism of hemorrhage production in tumors with this polysaccharide did not involve hemolysis of erythrocytes.

Some of the substances mentioned previously in this section were selected for trial in an effort to obtain information on possible mechanisms of hemorrhage production in tumors. The experiments of Andervont and Shimkin (*17*) led them to postulate that bacterial filtrate, by lowering suddenly the ascorbic acid content of the tumor, weakens its fragile capillaries with resultant hemorrhagic extravasation. This view was based on their finding that ascorbic acid prevented the production of hemorrhage in tumors with bacterial filtrate; similar but less pronounced inhibi-

[12] *Personal communication.*

tion was obtained with cysteine. Their experiments were carried out with sarcoma 37 and with the crude filtrate from cultures of *Serratia marcescens* as the hemorrhage-producing agent.

However, in our hands, neither ascorbic acid nor cysteine inhibited hemorrhage. The same technique was employed except that even smaller doses of the hemorrhage-producing agent were used. The injection of large doses of ascorbic acid or of cysteine was therefore expected to exert a more pronounced inhibition than these workers reported, but no appreciable influence on hemorrhage production was observed. The chief difference between the two sets of experiments was that Andervont and Shimkin employed the crude filtrate, whereas we employed a purified concentrate obtained from the filtrate. Whether this was responsible for the difference in the results is left for future work to determine. Similar lack of inhibition of hemorrhage production was observed with glutathione also. Shwartzman and Schick (*18, p. 160*) found that ascorbic acid failed to exert any effect on the production of the Shwartzman phenomenon with bacterial filtrate.

DISCUSSION

The relevant literature has been summarized in a preceding communication (*2*). The most recent discussion of the chemical nature of bacterial substances which produce hemorrhage in tumors is that of Zahl, Hutner, and coworkers (*19*), who were of the opinion that these agents are probably identical with a polypeptide component of certain complete endotoxin O antigens. In a later paper Hutner and Zahl (*20*) substituted "protein" for "polypeptide" in referring to their chemical nature.

The nitrogen content of our trypsin-digested potent preparations was about 2 percent, which is too low a value for a polypeptide. Furthermore, part of this small content of nitrogen has been accounted for by hexosamine and by the nitrogenous component of a phospholipid. Finally, the tests for proteins and polypeptides were essentially negative, and those for amino acids yielded only faintly positive results. In this connection, the findings of Freudenberg and Molter (*21*) are relevant. In studying the composition of the blood group A substance from urine, they detected some amino acids not of protein origin in the hydrolyzate; the substance itself was thought to be a polysaccharide.

The qualitative tests and quantitative data on carbohydrate substances showed that our preparations were largely polysaccharide in nature since about two-thirds of the material consisted of sugar residues. In addition, the components of a phospholipid were detected. The phospholipid moiety behaved as if it were present in firm combination and not in a loosely bound complex.

Zahl and Hutner (*22*) have reported that orally administered sulfanilamide is capable of inhibiting the hemorrhagic effect of about 16 minimum hemorrhage-producing doses of a fraction obtained from cultures of *Salmonella typhimurium*. They also reported (*20*) that sulfanilamide protected against 2 to 10 minimum lethal doses and that *p*-aminobenzoic acid interfered with the protective action of sulfanilamide.

Gerber and Gross (*23*) found that a sulfanilamide conjugate of a bacterial filtrate possessed both preparative and provocative potency in the Shwartzman reaction. A meningococcus culture filtrate was coupled with *p*-aminobenzenesulfonylacetylimide, and a highly colored conjugate was obtained. They concluded that chemical treatment of the bacterial filtrate in the synthesis of the conjugate apparently did not affect its ability to produce the Shwartzman reaction.

The active polysaccharide material from *Serratia marcescens* culture filtrate contained about 1 percent phosphorus in organic combination. Part, at least, of the phosphorus was present presumably as a component of a phospholipid. Whether any phosphate was present in combination with sugar residues is still an open question.

The potency was unaltered on standing at 37° C. However, the activity was destroyed at higher temperatures; the rate of destruction increased rapidly in the temperature range 60° to 100°. Treatment with acid (2 N hydrochloric acid) caused rapid loss of potency at 37°, while heating with acid at 80° produced more rapid destruction. These findings are in general agreement with those of Gardner, Bailey, and Hyde (*24*) who studied a toxic carbohydrate complex from *Salmonella enteriditis*.

However, they concluded that the hemorrhagic reactions produced in the Walker rat tumor 256 were due to the polysaccharide-free component of the complex. The low potency of their polysaccharide component may well have been a consequence of almost complete destruction of activity by heat and by acid, since their polysaccharide was obtained by a hydrolytic process involving heating at 100° C. for 4 hours in 0.2 N acetic acid. Detailed data on minimum hemorrhage-producing doses of the polysaccharide and of the toxic component obtained in this way were not given, but both had little potency, amounting in both cases only to about one one-thousandth that of the unheated material prior to hydrolysis. It would be worth while to attempt to devise a method of obtaining these components by a method which does not require such a high temperature or such strong acid.

Further work is required to establish whether the entire polysaccharide fraction from *Serratia marcescens* filtrate is required for potency, or whether it resides in some component of the complex. In all cases in which the "acetyl" group was completely removed, the potency was entirely destroyed. In a few instances, preparations with a low acetyl content were obtained which possessed high potency. It is not known at present whether this small residual amount of acetyl is essential for activity.

The production in this laboratory of hemorrhage in tumors with 0.02 mg. of the extract prepared by Antopol and Glick (*16*) was all the more unexpected inasmuch as high temperatures for many hours were employed in the preparation of the extract. If this material is chemically similar to the active polysaccharide fraction from *Serratia marcescens*, the potency might be expected to be greatly enhanced by avoidance of such high temperatures. The heat employed may have been responsible for the inconstant results obtained by Antopol (*25*) in producing the Shwartzman reaction with analogous extracts of sterile tumor tissue. The testing of the effect on tumors of Shwartzman-active extracts of sterile tumors is another aspect of the subject which appears to warrant attention.

Preparations obtained from a variety of bacteria have a strikingly selective action on tumor tissue. In the case of culture filtrates from at least two organisms, *Escherichia coli* (*26*) and *Serratia marcescens*, polysaccharide fractions, which gave negative results in tests for protein, have been obtained which induced hemorrhage in mouse sarcomas in doses less than 1 microgram. For convenience these materials are referred to as polysaccharide fractions, not because it has been demonstrated that the potency resides in the polysaccharide moiety but because these preparations are preponderantly polysaccharide in nature.

SUMMARY

The potent concentrate previously obtained from *Serratia marcescens* culture filtrate was found to retain its activity after digestion with trypsin. The minimum dose which produced hemorrhage in 50 percent of the tumors (sarcoma 37) was about 0.1 microgram. Elementary analysis of a number of preparations after tryptic digestion gave the following average results in percent: Carbon, 47.5; hydrogen, 7.1; nitrogen, 2.2; phosphorus, 1.1; acetyl, 2.2; ash, 3.5. No methoxyl was found. A single determination for sulfur gave a value of less than 0.2 percent. The values for nitrogen and "acetyl" were about half those obtained prior to digestion with trypsin.

The active material, upon optimum hydrolysis, yielded aldohexose, hexosamine, methylpentose, and the components of a phospholipid. About two-thirds of the material consisted of sugar residues, of which the major portion was aldohexose. The phospholipid was present in a firmly bound complex. The presence of neither polypeptide nor protein could be demonstrated.

The chemical behavior of the tryptic-digested material was investigated in a number of ways. The effects of the manipulations on the potency were determined with an improved bioassay technique, and the bioassay results correlated with the chemical findings.

The potency of aqueous solutions was not destroyed at 37° C. In the range 60° to 100°, potency was lost at rates which increased with the temperature. In the light of the heat stability of the material, the effect of acid and alkali on the potency was investigated at various temperatures, and the hydrolytic changes produced under these conditions were studied.

Acknowledgment is made of the generous gift of specimens of the following substances: extracts of mammalian tissues (Dr. William Antopol, Newark Beth Israel Hospital); heparin (Drs. A. F. Charles and L. B. Jaques, Connaught Laboratories, Toronto); α- and β- lignin sulfonic acids (Prof. Harold Hibbert, McGill University); coumarin derivative (Prof. K. P. Link, University of Wisconsin); and two azo dyes, Pontamine Sky Blue 6BX and Pontamine Fast Pink BL (DuPont Co.).

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Chemical Treatment of Tumors. VIII. Ultracentrifugal and Electrophoretic Analysis of the Hemorrhage-Producing Fraction from *Serratia marcescens (Bacillus prodigiosus)* Culture Filtrate

By HERBERT KAHLER, *senior biophysicist*, M. J. SHEAR, *principal biochemist*, and J. L. HARTWELL, *chemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

Some of the physicochemical properties of the material, prepared by Shear and Turner (*1*) from filtrates of *Serratia marcescens* (*Bacillus prodigiosus*) cultures, which produced hemorrhage in mouse tumors in doses of less than 1 microgram, were summarized in an earlier communication (*2*). This material was further purified by digestion with trypsin (*3*) and was shown to be polysaccharide in nature. The present paper reports the results of ultracentrifugal and electrophoretic analyses of three previously described batches (K–15c, P–2, and P–3) of the trypsin-digested polysaccharide fraction. For convenience, these preparations are designated in this paper as A, B, and C, respectively. They contained from 0.68 to 1.5 percent nondialyzable solids and produced hemorrhage in mouse sarcoma 37 in 50 percent of the mice with doses as low as 0.1 cc. of 1 : 10,000 dilution. These end-point doses contained about 0.1 microgram of the active polysaccharide fraction.

Information was sought concerning (1) the homogeneity and size of the particles as determined by ultracentrifugal and diffusion analysis, (2) the homogeneity and character as determined by electrophoretic analysis, and (3) the possible separation of this fraction into active and inactive components.

METHODS[1]

Ultracentrifugal Analysis

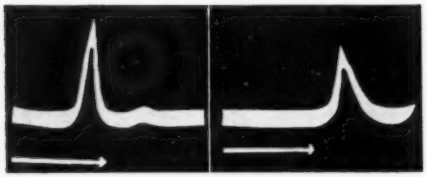
For ultracentrifugal analysis the polysaccharide solutions were brought to the desired pH with acetate or phosphate buffer. Preparation B gave a heavy precipitate when the ionic strength of added salts exceeded 0.1. The salt concentration was therefore usually kept lower than is the common practice in such work.

The solutions in the optical cell of an air-driven type ultracentrifuge were maintained at 20° ± 0.5° C. with a water-cooled jacket and an atmosphere of hydrogen. The Thovert - Philpot - Svennson optical system was used for obtaining the photographs. The sedimentation rates, expressed in Svedberg units (*s*), were corrected to standard conditions at 20°. The partial volume of the polysaccharide was found to be about 0.75.

Electrophoresis

The electrophoretic apparatus consisted of the conventional Tiselius cell with lenses of long focal length, the mechanically coupled Schlieren diaphragm and plateholder of Longsworth, and a bath maintained within 0.01° of 5° C.

[1] *A more complete description of the physical equipment is given by Kahler and Bryan* (4).



A *B*

FIGURE 1.—Tracings of photographs of preparation A in the ultracentrifuge (neutral solution; 0.2 N NaCl). *A*, Showing one major and one minor component. The arrow indicates the direction of sedimentation; B, Showing the appearance of the major component after the minor component had been completely sedimented.

The polysaccharide solutions, after being brought to the desired pH and ionic strength by dialysis for several days against suitable buffer solutions, were put into the lower half of the cell and the dialyzates into the upper half. In determining mobilities, the current was measured with a standardized milliammeter, and the conductivity with a Jones bridge. The values were corrected to 0° C.

DIFFUSION

For the determination of the diffusion constants, a cell of the type designed by Neurath was used. It was maintained at about 5° C. to minimize convection currents caused by slight temperature changes. The diffusion constants were calculated from the relation involving the areas under the curves and their maximum height.

BIOASSAY

The bioassays were carried out according to the method described in a preceding paper (*5*).

TABLE 1.—*Ultracentrifugation and diffusion, and electrophoresis of three preparations of the hemorrhage-producing polysaccharide fraction of* Serratia marcescens

ULTRACENTRIFUGATION AND DIFFUSION

Preparation	Concentration	Composition of buffer solution	pH	Sedimentation constant, 20° C. (c. g. s. units)	Electrophoretic mobility at 0° C. (cm.²/volt sec.)	Diffusion constant, 20° C. (cm.²/sec.)	Remarks
A	*Percent* 1.1	0.2 N NaCl	(1)	32.6 x 10^{-13}			Major component (fig. 1, *A* and *B*).
				63.7 x 10^{-13}			Minor component (fig. 1, *A*).
B	.33	Phosphate, 0.05μ	7.4	49.9 x 10^{-13}			Major component.
	.26	Acetate, 0.1μ+0.2 N NaCl	4.1	70.6 x 10^{-13}		0.89 x 10^{-7}	Material not salted out (fig. 2).
C	1.5	Phosphate, 0.1μ	7.4	63 x 10^{-13}			Major component (fig. 5, *A* and *B*).
				69 x 10^{-13}			Minor component (fig. 5, *A* and *B*).
	1.35	do	7.4	133 x 10^{-13}			1 day later (fig. 5, *C*).
	.5	do	7.4	234 x 10^{-13}			2 days later.
	.75	Acetate, 0.1μ	4.4	344 x 10^{-13}			Aggregation during run.
	.75	do	4.4	537 x 10^{-13}			2 hours later.

ELECTROPHORESIS [2]

B		Acetate, 0.1μ	4.1		−2.59 x 10^{-5}		Figure 3, *A* and *B*.
		Phosphate, 0.07μ	6.8		−2.66 x 10^{-5}		Prior to salting out (fig. 4, *A*).
		Phosphate, 0.13μ	6.8		−2.73 x 10^{-5}		Material not salted out (fig. 4, *B*).
		Phosphate, 0.07μ	6.8		−2.78 x 10^{-5}		Material salted out (fig. 4, *C*).
C		Phosphate, 0.1μ	7.4		−2.47 x 10^{-5}		Figure 6, *A* and *B*.
		Acetate, 0.1μ	4.4		−.19 x 10^{-5}		Major component.

[1] Approximately neutral.

[2] For each solution, under the conditions specified, there was little difference in the appearance of the boundary ascending into the buffer compartment and of that descending into the polysaccharide compartment of the electrophoresis cell. In each case the mobility given is the mean of the values for the ascending and descending boundaries. The photographs shown in the figures are for the ascending boundaries

RESULTS

The results are summarized in table 1 which shows appreciable differences in the physical characteristics of the different batches. Preparation A gave the lowest sedimentation rate; the major component (fig. 1, *A* and *B*) yielded a value of 32.6 *s*. A smaller component, with about twice this rate (63.7), was also obtained.

Preparation B, as stated previously, partially salted out with buffer solutions stronger than about 0.1μ.

To obtain sedimentation and diffusion constants to which complete corrections could be applied, it was desirable to use a buffer with an ionic strength greater than 0.1. Preparation B was accordingly dialyzed against a buffer solution composed of 0.1 N acetate (pH 4.1) and 0.2 N sodium chloride, and the sediment removed by centrifuging at 20,000 g for one-half hour. The sedimentation (fig. 2) and diffusion constants were determined on the supernatant solution to obtain an approximate idea of the molecular weight. A value of 70.6 x 10^{-13} c. g. s. units was obtained for the sedimentation constant, and of 0.89 x 10^{-7} cm.^{2}/sec. for the diffusion constant. From these values, it was calculated that the molecular weight was approximately eight million and that the frictional ratio ($f : f_o$) was 1.8. On the assumption of a molecule with the shape of a rotational ellipsoid, Perrin's formula gave values for the ratio of the long to the short axis of 16 for an oblong ellipsoid, and of 21 for an oblate ellipsoid.

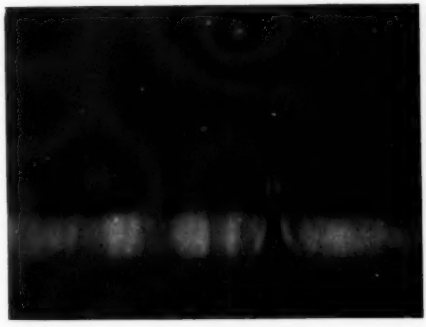


FIGURE 2.—Photograph of preparation B in the ultracentrifuge, after salting out half of the material, showing a sharp band with s_{20}= 70.6 (pH 4.1; 0.1μ acetate buffer + 0.2 N NaCl; molecular weight about eight million.

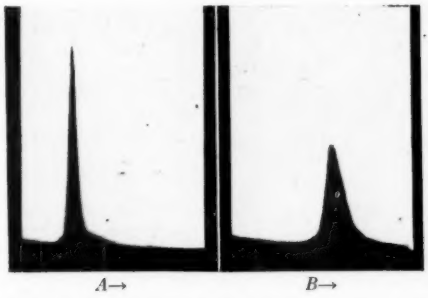


FIGURE 3.—Photographs of preparation B in the electrophoresis cell. (pH 4.1; 0.1μ acetate buffer; mobility, -2.59×10^{-5} cm.^{2}/volt. sec). *A*, At the end of 1 hour; *B*, At the end of 2 hours.

By electrophoresis, preparation B was found to be negatively charged; the mobility varied from -2.6 to -2.8 units (table 1) depending on the pH and on the salt strength. (In earlier experiments with material not purified by tryptic digestion no change in the sign of charge was found between pH 2 and 10.) The shape of the bands indicated the presence of only one major component (figs. 3 and 4).

Preparation C appeared to be labile in the ultracentrifuge, as judged by the tendency to form large aggregates on standing 1 and 2 days in salt solutions of 0.1μ (part of the time at room temperature). Preparation C at pH 7.4 gave sedimentation rates varying from 63 to 234 *s* depending on conditions (fig. 5), but the tendency to salt out at neutral pH was not so evident as in the case of preparation B. At pH 4.4, in acetate buffer, the sedimen-

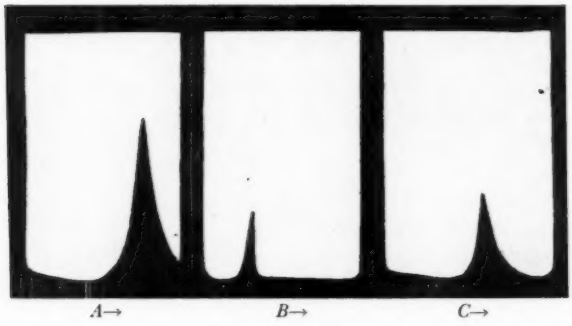


FIGURE 4.—*A*, Photograph of preparation B in the electrophoresis cell, prior to salting out. (pH 6.8; 0.07μ phosphate buffer); *B*, photograph of the supernatant solution, in the electrophoresis cell, obtained on salting out part of the material in preparation B (pH 6.8; 0.13μ phosphate buffer); *C*, photograph in the electrophoresis cell of the component salted out of preparation B at pH 6.8 with 0.13μ phosphate buffer (pH 6.8; 0.07μ phosphate buffer).

tation values rose to over 500 *s*, showing rapid aggregation. The electrophoretic mobilities of preparation C, which was also negatively charged (fig. 6), changed rapidly with pH in contrast with preparation B, in which there was little change with pH.

These preparations of the active polysaccharide fraction were found to be relatively homogeneous from the point of view of these physical properties. While the sedimentation values of all three preparations differed from one another, about 90 percent of the material in each was present at pH 7.4 as one component; the remainder was distributed in one or more minor components. By electrophoretic analysis similar results were obtained. Each preparation consisted mainly of one component which, during the migration in the standard Tiselius cell section, was not resolved further into well-defined constituents; however, the electrochemical characteristics of the main component varied from preparation to preparation.

The distribution of the hemorrhage-producing potency among the various components obtained on treating the active polysaccharide fraction by these different procedures gave no indication of further concentration of potency in any component. Previous dialysis experiments (*1*, *3*) had shown that the potency in producing hemorrhage in mouse sarcomas resided in high-molecular-weight material. When a strong concentration of acetate buffer was added to preparation C, no salting out occurred; however, centrifuging at 20,000 g for 3 hours threw down most of the material and most of the potency. The molecular weight of the material sedimented in this way was calculated to be within the range of the molecular sizes of the components listed in table 1.

The mobility of the main component of preparation B in 0.07μ phosphate buffer at pH 6.8 was -2.66 units (fig. 4, *A*). When the phosphate was increased to 0.13μ, about half the material salted out. The mobility of the main component remaining in the supernatant solution was -2.73. The sediment was taken up in 0.07μ phosphate; the main component of this fraction was found to have a mobility of -2.78. Thus the main component in the sediment had about the same mobility as that in the supernatant solution. Bioassays showed that the supernatant solution and the sediment each had about half of the total origi-

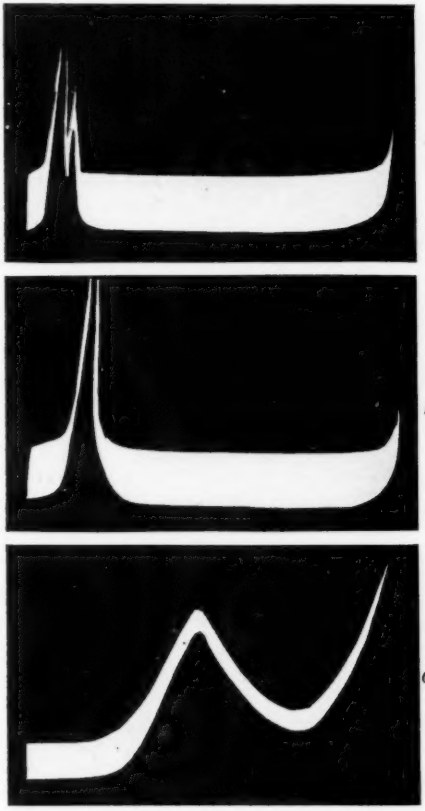


FIGURE 5.—Tracings of photographs of preparation C in the ultracentrifuge. *A*, Showing a major and a minor component (pH 7.4; 0.1 μ phosphate buffer); *B*, After centrifuging for an additional short period, showing the relative insignificance of the minor component; *C*, Same solution, after standing for 1 day.

nal potency; in terms of mouse tumor units (*5*) per milligram these two fractions had about the same potency. It therefore appeared that most of the potency in preparation B resided in this major component.

The major component of the concentrated solutions employed in these physicochemical studies varied in size and mobility from one preparation to another. It also varied in any given preparation with the salt concentration, pH, and time of standing. Despite these differences in the physical characteristics of the major component in the three preparations, the hemorrhage-producing activity was approximately the same.

Electron microscope photographs of several polysaccharide preparations were obtained through the cooperation of Dr. Gregory Shwartzman, of the Mt. Sinai Hospital, New York. Figure 7 shows the results obtained by Dr. Shwartzman with preparation B. The solution was freed from salts by dialysis against distilled water and was evaporated in a thin film at room temperature. This figure shows the presence of numerous large particles, fairly symmetrical in shape, with molecular weights ranging between three and thirty million (our estimate).

It has been found (*1*) that residues dried at ordinary temperatures and pressures, in contrast with material dried by lyophilization, lost both solubility in water and hemorrhage-producing potency. It remains to be seen what the electron microscope will show with material dried in a manner which does not destroy the potency.

FIGURE 6.—*A*, Photograph of preparation C in the electrophoresis cell (pH 7.4; 0.1μ phosphate buffer); *B*, Same as *A*, at the end of the experiment.

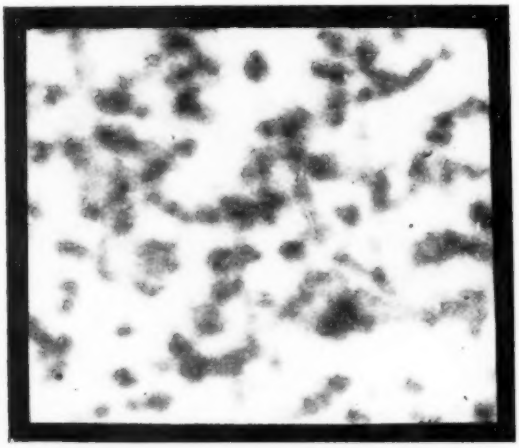


FIGURE 7.—Electron microscope photograph of preparation B, dried at ordinary temperature and pressure. Total magnification × 100,000.

COMMENT

As stated in the introduction, these experiments were carried out to ascertain how homogeneous these preparations were and, if found to be heterogeneous, to employ ultracentrifugal sedimentation and electrophoresis to separate the different components. Inasmuch as several kinds of polysaccharides may be elaborated by one organism, it had been anticipated that the active polysaccharide fraction from *Serratia marcescens* might consist of a considerable number of components. Contrary to our expectations, however, each preparation of this polysaccharide fraction was found to be relatively homogeneous.

In each of the three preparations the bulk of the material consisted of one major component, and the bulk of the potency was apparently associated with this component. In each case, the particles had a molecular weight in the range of one to ten million. Evidence of aggregation was obtained in buffer solutions of relatively low ionic strength, and the tendency to aggregate, which may account for the variations in the different preparations, appeared to depend on the salt concentration, pH, and time of standing in the salt solutions. The values reported are to be considered, therefore, as descriptive of these particular preparations under the conditions specified rather than as characteristic constants.

In view of the homogeneity of these preparations, it is considered that investigation of the physiologic effects of this material on the living animal would provide information on the action of the hemorrhage-producing agent which would not be complicated by accompanying effects of other biologically active, but nonspecific, polysaccharide contaminants.

SUMMARY

Analyses of three preparations of the polysaccharide fraction from *Serratia marcescens* (*Bacillus prodigiosus*) culture filtrates which produces hemorrhage in mouse sarcomas were carried out in the ultracentrifuge and in the electrophoretic apparatus. The sedimentation constant of the major component at pH 7.4 varied in the three preparations studied. With one fraction at pH 4.1 (0.3μ) the diffusion constant was found to be 0.89 x 10^{-7} cm.^{2}/sec. and the sedimentation constant at 20° to be 70.6 x 10^{-13} c. g. s. units.

These two values gave a molecular weight of about eight million under these conditions. The shape of the particles was markedly nonsymmetrical; the long to short axis ratio of an equivalent rotational ellipsoid was calculated to be 16 or 21, depending on the type of ellipsoid assumed.

In each preparation, about 90 percent of the material distinguished by electrophoresis or sedimentation was in one component.

The hemorrhage-producing activity resided in the readily sedimentable material. Salting-out tests indicated that the major component carried most of the activity.

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